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IN VITRO CHEMICAL CARCINOGENESIS AND CO-CARCINOGENESIS
IN HUMAN CELLS INITIATED BY HYDRAZINE AND
POLYNUCLEAR COMPONENTS OF JET FUEL

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Examination of the carcinogenic activity of selected hydrazine, polynuclear hydrocarbons and/or napthylamines on human cells was evaluated on human foreskin cell populations in vitro. We also evaluated the interaction of these compounds with feline oncornaviruses (ST-FeSV). To date we understand how this system can be used to evaluate the carcinogenic activity of the above suspected carcinogens. In Table 1 Segment 2, we have listed the chemicals evaluated in this system for their carcinogenic activity. Table 4 presents characteristics of the
(continued)

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Block 20 - Abstract (continued)

transformed cells in early, transitional and late stages of the carcino-
genic process. The content to Table 2 Segment 2 summarizes the comparative
aspects of the carcinogenic activity and cocarcinogenic activity of the
chemicals. In Table 2 Segment 2 it was found that RDDP activity was absent
in the carcinogen transformed cells. We can conclude from these data that
the induction of the carcinogenic process is not a consequence of indogenous
oncornavirus activation. These data presented to date illustrate that
human cells can be used to evaluate carcinogens (Segment 1) and cocarcino-
gens (Segment 2) on human cells.

Final Report to

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Directorate of Life Sciences
Bolling A.F.B., D.C. 20332

Title: IN VITRO CHEMICAL CARCINOGENESIS AND CO-CARCINOGENESIS

IN HUMAN CELLS INITIATED BY HYDRAZINE AND

POLYNUCLEAR COMPONENTS OF JET FUEL

Inclusive Dates of Report:

    July 1, 1977 - June 30, 1980

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Segment 1

Final Report Summary FY 1979

Segment 2

Segment 1

1. a) Work Scope: - 1977

   1. Examine the cytotoxic and carcinogenic potential of hydrazine and polynuclear hydrocarbons, componets of jet and rocket fuel on human cell populations, in vitro.

   2. Investigate damage to cellular DNA by studying DNA repair synthesis and alterations in collular associated DNA directed DNA polymerase activities.

   3. Investigate the post-translational responses to the cellular insults reflective of cytotoxic and carcinogenic responses.

   4. Investigate, with oncogenic DNA and RNA viruses, whether the suspect carcinogens act synergistically with these viruses to enhance virus directed transformation of human cells.

   5. Determine whether these chemicals damage cellular DNA, providing additional sites for virus integration.

The chemicals selected for investigation by Air Force representatives, Colonel V. Carter, Drs. K.C. Back, Berry and Ms. Marilyn George and The Ohio State University Research Foundation representatives Drs. George E. Milo and James Blakeslee were the following

α-$\emptyset$-napthylamine (-1-)
β-$\emptyset$-napthylamine (-2-)
α -napthylamine (-1-)
β -napthylamine (-2-)
monomethyl hydrazine
1,2-dimethyl hydrazine
hydrazine
symmetrical (1,1-) dimethyl hydrazine

We have also received some analogues of the arylamines that have been inserted into the regimen because they represent metabolities and or non-active analogues found in biological systems in response to treatment with the parent napthylamines.

Our interest in these agents is because, hydroxyl-arylamines have been shown to function as bladder carcinogens in dogs, (Kadlubar, et al., 1978). Apparently, the napthylamines are activated by activating enzymes at othe sites in the animal, possibly the liver, and the hydroxyl derivatives glucuronated and transported to the bladder.

Therefore the following analogues were evaluated.

N-OH-1-Napthylamine
N-OH-2-Napthylamine
α-NO_2-napthylamine
β-NO_2-napthylamine
4-amino biphenyl

The system used in Segment I treated with these aforementioned compounds were fibroblasts isolated from human neonatal foreskins taken from the hospital at random. The system used for Segment II was a Detroit 550, a cell line developed from a single human foreskin. The viruses selected to be used in this study were SV-40 and FeSV.

The long-range goal of these studies will be to develop a human in vitro assay system that can be used to screen chemicals for potential carcinogenicity within a short period of time. Secondly, to understand how these chemicals act to induce carcinogenesis. Interrelated with this effort will be the use of viruses in the system to investigate the stages in the carcinogenesis process induced by the virus and to study the interaction between the human cells, transforming virus and suspect chemical carcinogens.

b)   In 1977 our progress in Segment 1.

1. Cytotoxicity curves for 6 compounds was completed.
2. Acceptable indices for the different stages of the transformation process were developed; i.e. Morphological indices, enzyme markers, growth in soft agar, production of tumor in the mice.

II. a) Work Scope: 1978

A. To examine the cytotoxic effects of the polynuclear hydrocarbons and hydrazines in jet fuel have on relative cloning efficiencies of human cells in vitro,
B. To score the cultures for morphologically abnormal clones, (morphological transformation).
C. To serially subpassage the abnormal colonies beyond their normal lifespan and then clone in soft - agar followed by inoculation of a bolus of cells into a xenogeneic immunologically suppressed host to test for the malignant potential.
D. To synchronize human cells in vitro and treat synchronized cells with suspected chemical carcinogens during different phases of cell cycle, (G_0, G_1, S, G_2, M).
E. To investigate changes in DNA unscheduled repair kinetics after treatment of the cells in G_0 and G_1 cell cycling phases of the cell cycle.
F. To study changes in DNA directed DNA polymerase I and II activity after treatment of the cells with the carcinogens during the time when DNA repair is taking place in unsynchronized and synchronized cell populations.

b) Progress 1978-1979.

It has been found that human cells can be transformed in vitro by a number of laboratories in the U.S. (Kakunaga, T., at N.C.I., Kakunaga, Cold Spring Harbor Symposium pg. 1537-1548, 1977; Kakunaga, T. Proc. Nat. Acad. of Sci., 1978; Milo, G., and Dipaolo, Nature 115:130-132, 1978). At present other laboratories have been successful also in learning how to transform human cells, (Vogelstein at John Hopkins: McClosky at Northrop laboratories in Research Triangle Park: Maher at M.S.U. at East Lansing Michigan;

Zimmerman at Harvard University). The procedure we have determined is
effective in studying chemical carcinogen induced transformation was
presented in the progress report for the periods covering July 1, 1977 to
March 1, 1978. The cytotoxicity graphs reported on last year have been
expanded upon. The data accumulated at that time was by trypan blue dye
exclusion, (Annual progress report 1977-1978 Section IX page 83). We now
have extended those obsevations to a more sensitive procedure. However,
the second procedure, cloning studies, takes up to three weeks to complete
the experiments. Cytotoxicity data accumulated by this means has permitted
us to more rigidly define the "transformation dose" to be used in the
transformation treatment regimen. (Table 1). We have expanded these
results to include carcinogenicity data, (Table 1). The procedure used to
determine carcinogenicity data will be presented in the text of this years
report, (1979).

III. b) Progress in 1979

Table 1

Cytotoxicity Data (E.D. 50)

Suspect Chemical Carcinogen	Concentration ug/ml	Carcinogenic*
Benzo (a) Pyrene	>10	+
Benzo Pyrene- 8.diol 9,10 epoxside I (anti)	0.034	+
α-napthylamine	65	+
β-napthylamine	10	+
N-Ø-α-napthylamine	28	+
N-Ø-β-napthylamine	23	+
N-OH-α-napthylamine	2	+
N-OH-β-napthylamine	1.7	+
α-NO$_2$-napthylamine	42	-
β-NO$_2$-napthylamine	33	-
monomethylhydrazine	62.0	-
U.D.MH (1,1 DMH)	N.T.	+
1,2 DMH	N.T.	-
hydrazine	35	+
Methylazoxymethanol	0.005	-
bis UDMH (tetrazine)	50.0	+
phenylhydroaine	16.00	-
Methylazoxymethanol acetate	3.6	+

*Carcinogenicity is defined as the ability of the transformed cell
populations to produce a tumor in a nude mouse 4-6 weeks after inoculation
of the treated cells.

c) Other objectives and progress achieved in 1978-1979 are as follows:

I. We developed a protocol for evaluating suspect chemical or physical
carcinogens on human cells, (Figure 1). Once Stage 1 and 2 are complete we
then begin stage 3, TREATMENT PROTOCOL. Figure 2 illustrates how we treat
human cells in vitro to induce a transformation event.

The induction stage is composed of substages, i.e. activation and/or direct action of the carcinogen. Carcinogens requiring activation such as Benzo(a)Pyrene are added at 24 hour and left on until hour 44. Carcinogens not requiring activation such as B[a]P-diol-epoxide I (anti) or N-OH-napthylamines were added to the cells in early S (33 hour-34 hour).

Once the induction stage is complete we have determine that a progression of events (time) must elapse before different stages of expression of the transformed phenotype will be expressed (Figure 3).

Under each stage of expression, (Figure 3) we have listed indices that have retained a high correlation of reliability for 23 different carcinogen treated populations.

Figure 3

Table 1

Indices used to determine the transformed state of carcinogen treated normal human cells in vitro

1. Cell growth from low cell densities after 1:10 splits. (Early)
2. Shortened population doubling times. (Early)
3. Indefinite extension of lifespan. (Late)
4. Loss of density dependent inhibition when high cell densities were reached. (Early)
5. Cells in colonies seeded at low cell density from criss-cross disoriented piled up non-contact inhibited colony morphology. (Early only)
6. Cells exhibit change from fibroblastic shape in morphology to short polygonal multinucleate architecture. (Early only)
7. Cell growth in air atmosphere without CO_2 enrichment at 37°. (Early)
8. Cell growth in 1% fetal bovine supplemented 1x growth medium. (Early)
9. Cell growth at 41° up to 144 hours while normal cells did not survive a 24 hour treatment. (Early)
10. Cell growth in 0.3% agar supplemented with 1x growth medium low in calcium. (Transitional).
11. Cell growth in 1% agarose supplemented with 1x growth medium. (Transitional).
12. Different agglutination pattern of the transformed cells compared to normal untreated cells (Wheat germ agglutination). (Early)
13. Differential tolerance to selective chemical compounds and complexes indicative of the transformed state; e.g., heparin tolerance, ouabin sensitivity, dextran sulfate tolerance. (Transitional)
14. Differential response of transformed cells to tumor cell lysates. (Transitional)
15. Growth in nu/nu mouse, 4-6 weeks. (Late)
16. Growth on a embryonic chick skin organ culture explant (3 days). (Late)

Other indices are listed in Table

1. Index number 16 is presently under evaluation. To date we have a 100% correlation with mouse tumor data.
2. Early events in the transformation process are sometimes difficult to detect and in vitro indices are used to differentiate transformed cell populations from normal cell populations. The agglutinin we chose in this case was wheat germ lectin. The following results were obtained for the following transformed cell populations, Table 2,3.
3. One of the procedures we use to select for transformed cell populations before injection into nude mice is to serial passage the cells through soft agar. If they passage through soft agar twice, they will, as a rule, grow in nude mice, Table 4.

We have defined an adequate number of indices that can be associated with changes associated with the transformation process at the cellular level.

Table 2. - Resistance of Normal of Transformed[a] Cells to Heparin or Dextran Sulfate.

Treatment	Passage Level	Cloning Efficiency	Heparin (µg/ml)				Dextran Sulfate (µg/ml)			
			400	100	25	6	80	20	5	1
None	32	3	7[b]	15	27	101	2	8	24	81
AF-B$_1$	23	7	3	24	73	95	4	16	73	94
N-Ac-AAF	23	6	4	47	86	83	3	19	68	104
PrS	25	6	4	55	95	110	4	28	93	112
4-NQO	36	5	7	57	58	68	5	5	8	70
ß-PL	46	8	19	84	98	100	8	10	18	74
1-NA	53	8	40	89	93	84	5	9	28	97
2-NA	60	9	24	108	127	130	1	2	7	122

[a]Human cell populations transformed by N-Ac-AAF, PrS, 4NQO, ß-PL, 1-NA, 1-NA or Af-B$_1$ serially passaged to PDL 20. The transformed cells were passaged through soft agar (0.33%), repopulated and used during the transitional stage. The control cells (noncarcinogen treated) were serially passaged to comparable PDL.

[b]Percent survivors, based upon the cloning of untreated cells. N.D.=not done.

Table 3. - Resistance of Normal of Transformed[a] Cells to KB Cell Lysate.

Treatment	Passage Level	Cloning Efficiency	KB Lysate (Cell Equivalent X 10^5)					
			20.0	10.0	2.0	1.0	0.6	0.25
None	5	6	10^6	9	103	94	100	98
None	32	3	N.D.	N.D.	32	68	85	96
AF-B$_1$	23	/	37	78	51	91	85	81
N-Ac-AAF	23	6	8	64	83	102	101	99
PrS	25	6	33	67	71	95	106	102
4-NQO	36	5	N.D	N.D.	60	76	81	97
β-PL	46	8	N.D.	N.D.	85	102	105	95
1-NA	53	8	N.D.	N.D.	98	97	97	89
2-NA	60	9	N.D.	N.D.	112	121	93	115

[a]Human cell populations transformed by N-Ac-AAF, PrS, 4NQO, β-PL, 1-NA, 1-NA or Af-B$_1$ serially passaged to PDL 20. The transformed cells were passaged through soft agar (0.33%), repopulated and used during the transitional stage. The control cells (noncarcinogen treated) were serially passaged to comparable PDL.

[b]Percent survivors, based upon the cloning of untreated cells. N.D.=not done.

Table 4. - Characteristics of Transformed Human Skin Fibroblasts During
Transition from the Early Periods of the Transformation Process
Through to Neoplasia.

Carcinogen	E.D. 50[a]	L.A.[b]	S.A.[c]	T.I.[d]
β-PL	13.0	N.D.[e]	14.0	3/4
AF-B$_1$	10.0	78	10.0	8/14
PrS	5.0	125	20.0	7/11
4-NQO	0.002	39	0.1	2/4
MNNG	0.5	39	1.0	3/5
EMS	10.0	N.D.	20.0	2/4
1-NA	65.0	19	1.5	2/9
2-NA	68.0	250	0.1	1/6
N-OH-1-NA	2.0	N.D.	30.0	1/8
N-OH-2-NA	1.7	N.D.	29.0	2/7
N-O-1-NA	27.5	19	5.1	3/16
N-O-2-NA	23.1	250	1.0	3/16
N-Ac-AAF	0.5	39	0	0/8
Hydrazine	35.0	19	9.2	4/8
UDMH	50.0	N.D.	5.1	4/8
MAMA	3.6	N.D.	900.0	2/16
B(a)P	10.0	39	1.0	6/10
MMS	0.1	2500	0	0/6
U.V.	40 J.m^{-2}	78	20.0	4/6
137Cs	100 r	39	13.1	3/7
Control	---	2500	0	0/10

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Legend - Table 4.

[a]The ED 50 of these carcinogens was determined prior to and following the induction phase of the transformation protocol. The Toxicity values presented here were obtained in the pretreatment phase.

[b]These values were obtained using wheat germ agglutinin (L.A.=lectin agglutination).

[c]Fifty thousand cells at PDL 20 were seeded into 0.33% agar (S.A.) supplemented with LoCal + 20% FBS, overlaid on a 2.0% agar base supplemented with RPMI-1629 +20% FBS. The colonies were counted after 21 days. Frequency as expressed in column c (S.A.) is the number of colonies formed 28 days after seeding per number of cells seeded. The values were normalized to one hundred thousand cells.

[d]The tumor incidence (T.I.) is expressed as a fraction: the numberator is the number of mice giving rise to tumors 0.8 - 1.2 cm in size, 4-6 weeks after the injection of 5×10^6 cells and the denominator is the total number of preirradiated (450 rats, whole body) mice injected with a given cell population.

[e]N.D.=Not determined.

Text Continued

The indices we have selected as of primary importance to use are presented in Table 4. Our reasons for deciding on these are: 1) The early stage in the process can be readily identified by altered lectin agglutination profiles. Moreover, recently these lectins are available with an F.I.T.C. label attached and the amount of binding can be quantified.

Moreover the growth-of-transformed cells in soft agar a measure of the state of anchorage independence can be quantified. Lastly, tumor production in the mouse can be quantified. The main drawbacks to the nude mouse is 1) positive tumor formation occurs in 4-6 weeks, however negative results are not scoreable within 1 year, 2) Variability in resistance to susceptibility in nu/nu mice from different backgrounds, 3) Interference in tumor takes by the presence of indigenous murine viruses, 4) cost of housing.

Included under this cover is a photograph of colonies (UDMH-transformed cells) growing in soft agar.

Colony(s) of UDMH transformed human skin fibroblasts 3 weeks after seeding growing in 0.33% agar. These colonies are removed and then placed in nude mice (Figure 5). After 6 weeks the tumors are excised (Figure 6B) and submitted for histopathology.

III. Presentations, Abstracts - FY-1979

1. Transformation of Human Cells by Chemical Carcinogens. Gordon Research Conference Symposuim speaker at Colby-Sawyre College, New London, New Hampshire 8/20-24/79.

2. Characteristics of Cytoplasmic Polynuclear Hydrocarbon Protein. R. Tejwani and G. Milo. Polynuclear Aromatic Hydrocarbon Symposium. Battelle Columbus Labs. 10/2-4/79.

3. Influence of the ionic character of N-OH arylamines on the in vitro transformation of normal human fibroblasts. J. Oldham, G. Milo and Fred Kadlubar. Presented at the Soc. Toxicol. Meetings 3/1980. Pbl. in Toxicol. Appl. Pharmacol.

4. Benzo(a)Pyrene metabolism by transformable human skin fibroblasts. R. Tejwani, R.W. Trewyn, and G.E. Milo. Fred. Proc. Abs. in Biochemistry 39, 2091, 1980.

5. Requirements for the induction of carcinogenesis in human cells for different classes of jet fuel componets. G.E. Milo, J.N. Oldham, and R. Tejwani. Presented at A.F.O.S.R. meeting in January 1980 at San Antonio, Texas.

6. Kinetics of Movement of Benzo[a]Pyrene into transformable and non-transformable Human Diploid Cells. R. Tejwani, R.W. Trewyn, and G. Milo.

IV. Papers Published FY-1979 Sponsored by A.F.S.O.R.

1. R. Tejwani, S. Nesnow and G. Milo (1980) Analysis of intracellular distribution and binding of benzo(a)pyrene in human dysloid fibroblasts. Cancer Letters 10:57-65.

2. G. Milo, G.A. Ackerman, and I. Nayes. (1980) Growth and ultra-structural characterization of proliferating human keratenocytes in vitro without added extrinsic factors. In Vitro 16:20-30.

3. I. Noyes, G. Milo, and C. Cunningham. (1980) Establishment of proliferating human epithelial cells in vitro from cell suspensions of neonatal foreskin. Tissue Culture Assoc. Laboratory Manual 5:1173-1176.

4. G. Milo and J. Blakeslee. (1980) Hydrazine and UDMH induced neoplastic transformation and feline sarcoma virus induced carcarcinogemic effect on human diploid cells in vitro. Proc. of the Ann. Conf. on Environ. Toxicol. 9:112-120.

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V. Papers In Press: 1980 Sponsored by A.F.S.O.R.

1. G. Milo, R. Olsen, S. Weisbrode, and J. McCloskey (1980) Feline sarcoma virus induced in vitro progression from premalignant to neoplastic transformation of human diploid cells. In Vitro.

2. G. Milo, S. Weisbrode, R. Zimmerman and J. McCloskey. (1980) Ultraviolet radiation induced neoplastic transformation of normal human cells in vitro. Chem. Biol. Int.

3. G. Milo and J. DiaPaolo (1980) Presensitization of human cells with extrinsic signals to induce chemical carcinogenesis. Internat. J. of Cancer.

VI. Papers Submitted 1980 Sponsored by A.F.S.O.R.

1. G. Milo, J. Oldham, R. Zimmerman, G. Hatch and S. Weisbrode. (1980) Phenotypic characterization of human cells transformed by chemical and physical carcenogens, In Vitro. In Vitro.

2. G. Milo, R. Trewyn, R. Tywani and J. Oldham. (1980) Intertissue variation in benzo(a)pyrene metabolism by human skin lung and liver in vitro. A.G.A.R.D. Symposium. Toronto, Canada.

3. R. Trewyn, W. Douglas and G. Milo (1980). Evaluation of Benzo(a)pyrene metabolites generated by human lung epithelial cells. Carcinogenesis.

Principal Experiments in Progress with Other Laboratories

We are presently concluding work with Dr. Fred Kadlubar at the National Center for Toxicological research on the activity of the amines and their deterivatives on the interaction of the amines and DNA. Another collaboration that will bear fruition presently will be our work with Dr. Allen Jefferies at the College of Physicians and Surgens Comprehensive Cancer Center at Columbia University. We are examining the relationship between chemical carcinogen induction and adduct formation. Our work with Dr. Donald Witiak is beginning to bear results. We are assisting his laboratory in two ways. First, we are studying the relationship between the hydrazine compounds and derivatives with the induction of carcinogenesis and adduct formation. Secondly, we are using the radiolable hydrazine carcinogens in our system to study the events that occur during the expression phase of carcinogenesis. This is being accomplish by studying the interaction of the hydrazines with the acid soluble histone and non-histone nuclear proteins. We have concluded the radiolabeling studies and are finishing up the studies designed to quantitate the changes in histones during the early and transitional stages of the carcenogenesis process.

Our work on the translocation of benzo(a) pyrene into the human cells in continuing. We have determined that the cytoplasmic protein complex is required to transport the BP into the nucleus. We have identified the protein complex to be a lipoprotein of a molecular weight value of 12,500. We are presently characterizing tyhe complex.

Principal Experiments and Time Schedule for the Main Objectives for FY-80

The objectives mentioned on page 12 in the 1979 annual report have been accomplished with the following exception(s). Due to the deletion from last years budget for D. L. Allred salary and supplies we deleted the E.M. work. Another change we have made in the interest of time for evaluating the neoplastic stage of the transformation process is to use a chick embryonic skin organ culture. The methodology for the organ culture was worked out in this laboratory. The chief advantage to using this system is a reduction in time to evaluate neoplastic transformation. We need only 3 days to evaluate neoplasica with the chick skin system whereas we need 4-6 weeks to evaluate tumor formation in the nude mouse, (Renewal Proposal 1980). In one of the manuscripts accepted in the International Journal of Cancer we have examined specific compounds for their modulating effects for presensitizing human cells to the subsequent induction of carcinogenesis in the presence of the chemical carcinogens. We have just completed carcinogen induction experiments on the human skin epithelial system. We will be publishing on this in the fall. Our work is going along well in our collaborative effort with Dr. Witiak.

We are continuing our experimentation on the studies examining changes in histone labeling patterns during the different stages of the carcinogenesis process.

IX. Support Data 16 Copies of each document.

Figure 1

PROTOCOL

SELECTION OF CARCINOGEN	→	CYTOTOXICITY INDICES	→	TREATMENT PROTOCOL
1		2		3
MORPHOLOGIC TRANSFORMATION INDICES	→	SELECTION OF TRANSFORMED CELLS	→	CRITERIA USED TO DETERMINE TRANSFORMATION
4		5		6
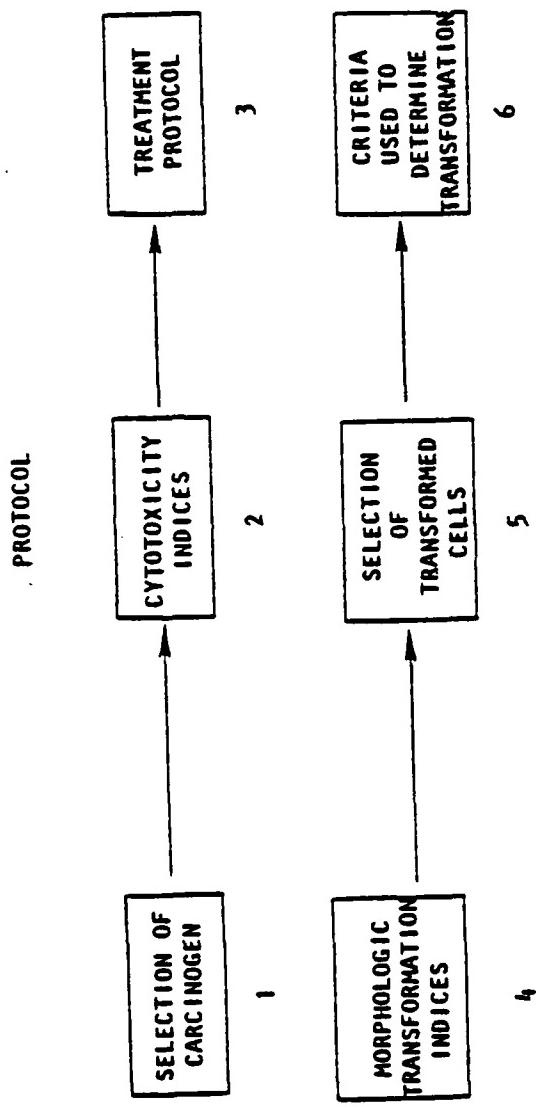
Figure 2

TREATMENT PROTOCOL
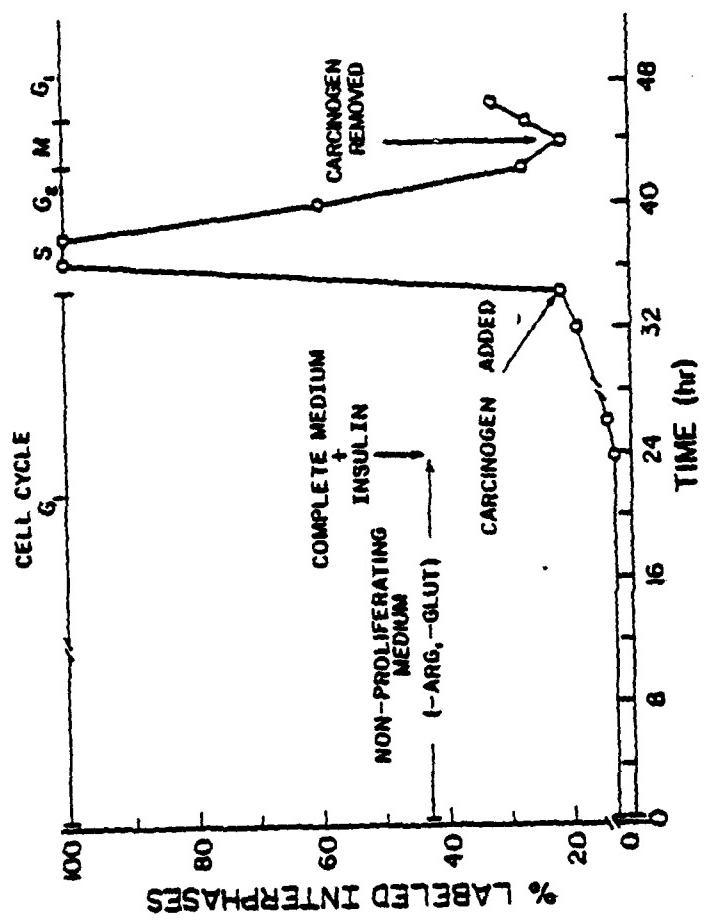
Figure 3

SELECTION OF TRANSFORMED PHENOTYPE

|——— EARLY ———|——— TRANSITIONAL ———|——— LATE ———|

Treatment
PDL
5

PDL
16-20

PDL
28-32

PDL
34-40

8 x Medium

0.33% Soft Agar

8 x Medium

LECTIN AGGLUTINATION
ABNORMAL COLONY FORMATION
GROWTH AT 41°C
GROWTH IN 1% SERUM
LOSS OF DENSITY DEPENDENCE

5 wks

GROWTH IN
SOFT
AGAR
KB CELL LYSATE
SENSITIVITY

8 wks

10 wks

5x10⁶ cells subcutaneously

TUMOR GROWTH
IN
NUDE MICE

14-16 wks

Figure 4

Figure 5

Characteristics of Cytoplasmic Polynuclear Hydrocarbon

Binding Protein

R. Tejwani and G. E. Milo

When proliferating human diploid foreskin cells were treated with ^{3}H-B(a)P _in_ _vitro_, the radiolabel was found associated with a cytoplasmic protein. Optimum binding of the ^{3}H-B(a)P occurred after twelve hours. Twelve hours later the radiolabel was localized in the nucleus. The ^{3}H-B(a)P was bound to a protein of molecular weight 12,500, as determined by Sephadex G-200 chromatography. Organic extraction and separation of the radiolabelled polynuclear hydrocarbon from the complex followed by high pressure liquid chromatographic analysis of the B(a)P fraction indicated that the parent B(a)P molecule , and not the polynuclear hydrocarbon metabolites, was bound to the protein. Non-proliferating cells, which contained inducible functional AHH activity, did not bind or transport the ^{3}H-B(a)P into the cytoplasm. The events associated with binding and movement of ^{3}H-B(a)P into the nucleus subsequently led to the induction of a carcinogenic response by the treated human diploid cell population.

INFLUENCE OF THE IONIC CHARACTER OF N-OH ARYLAMINES ON THE <u>IN VITRO</u> TRANSFORMATION OF NORMAL HUMAN FIBROBLASTS. James W. Oldham, George E. Milo and *Fred F. Kadlubar, Depts. of Vet. Pathobiology, Physiol. Chem. and the Comprehensive Cancer Ctr., Ohio State Univ., Columbus, OH, and the *National Ctr. for Toxicological Res., Jefferson, AR. Sponsor: <u>Roger Yeary</u>

Altering incubation conditions from a neutral to a slightly acidic pH causes an 18-fold increase in the <u>in vitro</u> binding of N-hydroxy-1-naphthylamine(N-OH-1-NA) to calf thymus DNA(Kadlubar <u>et</u>. <u>al</u>., Cancer Res. 38: 3628, 1978). Therefore, we studied the effect of pH on the <u>in vitro</u> transformation of normal human cells induced by N-OH-1-NA or N-OH-2-NA. Low passage human fibroblasts were blocked prior to the G_1/S interphase by amino acid deprivation, released and then treated during the early S phase of the cell cycle with 2.0 µg/ml N-OH-1-NA or N-OH-2-NA for 15 minutes(under argon) in serum-free medium at either pH 5.0 or pH 7.0. Affected cells were then selectively propagated in growth medium containing 8X nonessential amino acid and 2X vitamins. A comparison of the frequency of growth in soft agar(0.3%) demonstrated a 7-fold increase in N-OH-1-NA —induced bolus formation with cells treated at pH 5.0$(1:10^{2.88})$ over those treated at pH 7.0$(1:10^{3.72})$. Similarly, bolus formation from cells treated with N-OH-2-NA at pH 5.0$(1:10^{3.02})$ was 4-fold higher than those treated at pH 7.0$(1:10^{3.63})$. The conversion of the N-OH arylamines to an arylnitrenium ion or carbocation is favored at pH 5.0 and the acidic conditions normally found in the bladder lumen would also favor the formation of these electrophilic ions. Therefore, these results further implicate the N-OH arylamines as having a significant role in arylamine-induced urinary bladder carcinogenesis. (This work was supported in part by Air Force Office of Sci. Research F49620-77-C-0110)

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BENZO(a)PYRENE METABOLISM BY TRANSFORMABLE HUMAN SKIN FIBROBLASTS. Raman Tejwani*, Ronald W. Trewyn*, and George E. Milo. The Ohio State University, Columbus, Ohio 43210.

The metabolism of benzo(a)pyrene (BP) to reactive intermediates by microsomal mixed-function oxidases (MFO's) is considered to be important in the induction of carcinogenic events in mammalian cells. Human skin fibroblasts can be transformed in vitro by BP (Cancer Res. 38: 3026, 1978), so these cells are being used to study the role of MFO's in the transformation of human cells. Profiles of intracellular and extracellular metabolites of BP have been examined using high performance liquid chromatography to quantitate oxygenated products of the MFO's. After treatment, the primary intracellular (nuclear) polycyclic aromatic hydrocarbon (PAH) is the parent compound BP. Extracellular metabolites account for less than 10% of the added (0.1 uM) PAH after 24 hours, with phenolic derivatives representing the major (2%) oxygenated form. BP tetrols (diol epoxides), diols, and quinones account for less than 1% of the remaining metabolite. The limited generation of oxygenated intermediates by microsomal MFO's suggests that these enzymes play an alternate or indirect role in the transformation of human skin fibroblasts by BP. Other enzyme-derived, organic-extractable metabolites of BP, possibly methylated derivatives, are being evaluated for their role in the transformation process. (Supported in part by NIH R-01-CA-25901-01; Air Force F-49620-77-C-0110).

All compounds that are designated by code or initial letters must be identified adequately in the abstract, e.g., MJ-1999: 4-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide hydrochloride.

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Requirements for the induction of carcinogenesis in human cells for different classes of Jet Fuel Components. George E. Milo, James W. Oldham and Raman Tejwani. Department of Physiological Chemistry and Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210

Recent data reported at the Ninth Annual Environmental Toxicology Meeting held in Dayton indicated that hydrazine and UDMH could initiate early events in the carcinogenesis process in human cells leading to tumor growth of these cells in a suitable assay system. The tumors, when removed from the mice and analyzed in vitro by G-banding, carried karyotypic markers suggesting a change in the heterochromatin. Recent evidence indicates that compound requiring enzymatic activation such as benzo (a) pyrene (B (a) P) are specifically transported to the nucleus as parent B(a)P by a carrier protein complex. The B(a)P is then activated in the nucleus and the metabolites interact with the DNA. Results from adduct studies indicate that the 7,8-9, 10 ene diol epoxide I metabolite interacts with the guanosine residue in DNA. Other compounds such as N-OH arylamines that do not require enzymatic activation or specific transport into the nucleus need only a 15 minute treatment period when the cells are in S phase of the cell cycle to induce a carcinogenic response. This response is enhanced by treating at an acid pH where the formation of an electrophilic arylnitrenium is favored. Hydrazine and UDMH do not require activation and have a very short half life in the growth medium, approximately 2-5 minutes. Effective carcinogenic responses occur when the cells are treated during early S. Therefore we have identified specific requirements for activation, transport and molecular interaction that are different for each class of carcinogen.

Our immediate plans are to:

1) use chemical analogues of hydrazine or UDMH to study the mechanism of the carcinogenesis process

2) investigate the roles plasma membrane associated aryl-hydrocarbon hydroxylase (AHH) activation and nuclear associated AHH have in the detoxification and/or carcinogenesis process

3) continue characterizing the metabolite profiles and movement of BP into the cell

4) study events immediately following carcinogen localization in the nucleus, i.e. post-translational modification of histones and alterations in DNA polymerase activities.

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Kinetics of Movement of Benzo[a]pyrene into Transformable and Non-Transformable Human Diploid Cells

R. Tejwani, R. W. Trewyn and G. E. Milo

Benzo[a]pyrene [B(a)P], an environmental pollutant, can transform
low passage (<PDL 6) human skin fibroblasts in vitro (Cancer Res. 38:
3026, 1978). High passage cells (>PDL 20) are resistant to transformation
by this carcinogen. B(a)P binds differentially to cytoplasmic protein
complexes in the transformable and refractory cells (Cancer Letters,
in press). In the transformable cells, the movement of the B(a)P to the
nucleus is coincident with the optimum time for induction of carcinogenesis;
ie, 12-24 hours post-treatment. Therefore, low and high passage cells
were treated with [G-^{3}H]B(a)P for 12 hours followed by exposure to
unlabeled B(a)P for up to 96 hours. This pulse-chase experiment was
designed to follow the distribution of the B(a)P and/or B(a)P metabolites
in the cytoplasm, nucleus, and extracellular growth medium over the
course of 96 hours. High performance liquid chromtography profiles of
the intracellular and extracellular, ethyl acetate extractable fractions
demonstrated that >75% of the B(a)P remained unmetabolized in sensitive
and refractory cells. The uptake of B(a)P into the nucleus of the non-
transformable cells was approximately 50% less than the transformable
cells at most time points. Also, the non-transformable cells removed the
B(a)P from the nucleus to the extracellular environment more rapidly
over the 96 hr time period. The refractory nature of the high passage
cells may be explained by the differential binding of B(a)P to cytoplasmic
proteins, the diminished transport of B(a)P to the nucleus, and/or the
more rapid removal of B(a)P localized in the nucleus.

Supported in part by RO1-CA-25907 and A.F.S.O.R. F49620-77-C-0110.

ANALYSIS OF INTRACELLULAR DISTRIBUTION AND BINDING OF BENZO[a]PYRENE IN HUMAN DIPLOID FIBROBLASTS*

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SUMMARY

Previous work with low passage synchronized human foreskin fibroblast cell populations has indicated that benzo[a]pyrene (BP) can induce a carcinogenic event [3]. BP additionally has shown to damage DNA in logarithmically growing low passage cultures [9]. High passage cells, on the other hand, seem to be refractory to transformation by BP, even though this agent can induce DNA damage, similar to that seen in low passage cells. When low passage cells were treated with BP, the initial binding of the hydrocarbon was primarily to a cytoplasmic protein complex of molecular weight 12,500, while in high passage cells, a major portion of BP was bound to a protein complex of molecular weight 200,000. High-pressure liquid chromatography (HPLC) profiles of ethyl acetate extractable fractions from the BP-cytoplasmic protein complexes of low and high passage cells demonstrated that the majority of the BP remained unmetabolized. When nuclei were isolated from low and high passage cells prior to the HPLC analysis, the major component (90%) was again unmetabolized BP. The results suggest selective attachment of BP to different cytoplasmic protein

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Abbreviations: PNH, polynuclear hydrocarbon; BP, benzo[a]pyrene; HNF, human neonatal foreskin; HPLC, high pressure liquid chromatography; Buffer A, 0.02 M Na phosphate — 0.03 M Tris—HCl — 2.5 mM Na, EDTA — 0.5 mM DTT (dithiothreitol), pH 7.5; Buffer B, 0.01 M Tris maleate — 1 mM DTT — 3 mM Ca (Ac), — 2 mM Mg (Ac),, pH 7.5; BHT, butylated hydroxytoluene; BP-9, 10-diol, 9,10 dihydro-9,10-dihydroxy BP; BP-4,5-diol, 4,5-dihydro-4,5-dihydroxy BP; BP-7,8-diol, 7,8-dihydro-7,8-dihydroxy BP; BP-11, 12-diol, 11,12-dihydro-11,12-dihydroxy BP.

complexes of logarithmically growing human diploid fibroblast cells dependent on the passage level of the cells.

INTRODUCTION

Polynuclear hydrocarbons (PNH) are a class of molecules which must be activated to reactive metabolites in order to function as mutagens or carcinogens. This activation involves the conversion of polynuclear hydrocarbons (PNH)[4], such as BP, to dihydrodiols, epoxides, phenols, quinones and water soluble conjugates [4—6,12,13]. The 7,8-dihydrodiol-9,10-epoxide of BP has been shown to be the major reactive metabolite bound to DNA [14]; BP 7,8-dihydrodiol-9,10 epoxide (anti) deoxyguanosine is the major DNA adduct formed in human and bovine bronchial explant tissue [7]. The formation and cellular processing of this covalent DNA adduct is suspected to be a crucial event in BP-induced carcinogenesis.

Earlier reports from our laboratory have shown that BP absorbed into human neonatal foreskin (HNF) cells in culture and first accumulates in the cytoplasm [3]. Labelling of the cells with L-[4,5-^{3}H]leucine before treatment with [7,10-^{14}C]BP indicated that the PNH is initially bound to a cytoplasmic protein complex (unpublished data). Distribution of the BP into the nucleus of treated cells occurs 12 h later [3]. Moreover, BP treatment of HNF cells neoplastically transforms them at passage level 5 (low passage cells), but does not transform cells above passage level 10 (high passage cells) [8]. Optimum transformation is observed when low passage cells are treated with BP 12—24 h prior to entering the S phase; this treatment causes 3.0 breaks/10^8 daltons of DNA [9]. The time required for optimum BP-induced DNA damage coincides with the specific time period in which optimum BP enhancement of focus formation occurs in SV-40 infected transformable cells, i.e., treatment of the cells with BP 12—24 h prior to infection has been shown to enhance focus formation 2-fold [11]. Since SV-40 (viral) DNA does not need to replicate in order to be integrated into the host cell DNA, these observations support the concept that parent (unmetabolized) BP is directly involved in the transformation process within the nucleus. This report presents studies on the nature of association of BP with the cytoplasmic protein complex and nuclei from low and high passage cells, 12—24 h following the initiation of treatment.

MATERIALS AND METHODS

Preparation and isolation of BP-cytoplasmic protein complex

All extraction procedures were carried out under argon and red light to reduce photoxidation and autoxidation.

Passage 5—25 HNF cells were grown, serially subpassaged, and treated with BP as described earlier [3]. After seeding 48—72 h (40—60% cell confluency), the growth medium was replaced with a carcinogen-supple-

mented medium containing 0.125—26 μM [G-^{3}H]BP (16—40 Ci/mmol).
After 12 h of treatment, the cells were harvested and washed. The 650 $\times g$
cell pellet [3] was homogenized in 2 ml of Buffer A and centrifuged at
100,000 $\times g$ for 1 h. The cytoplasmic fraction was partitioned with dextran-
coated charcoal in Buffer A, and 2 ml (0.2—0.3 mg/ml protein) of the
[G-^{3}H]BP protein complex was applied to a Sephadex G-200 column
(58 × 0.9 cm). Elution of 0.5 ml fractions was carried out with 50 ml of
Buffer A at a flow rate of 15 ml/h. An aliquot of each fraction was removed
and the radioactivity assayed in a Packard Tri-Carb liquid scintillation
counter at a tritium counting efficiency of 38%.

HPLC of [G-^{3}H]BP metabolites

Preconfluent cells were exposed to 0.072 μM [G-^{3}H]BP (1 mCi/ml,
27 Ci/mmol) for 12 h and a 100,000 $\times g$ fraction was prepared as described
above. For the nuclear metabolite profiles, cells were treated with 26 μM
[G-^{3}H]BP (1 mCi/ml, 27 Ci/mmol) for 24 h, and nuclei were prepared by
a modification of the procedure of Chaveau et al. [2]. The nuclear pellet
was suspended in Buffer B and contrast-interference Nomarski microscopic
examination of the nuclear suspension indicated a 35% recovery of nuclei.

Extraction of either the BP-cytoplasmic protein complex or the nuclear
fraction was completed with 3 vols. of ethyl acetate in the presence of
0.8 mg/ml BHT; the organic phase was passed over anhydrous sodium sulfate,
filtered, dried under argon and dissolved in 0.5 ml of acetone/methanol
(2 : 1 v/v). Aliquots were removed for counting and the remaining sample
was dried under argon and stored at −90°C. The sample was reconstituted
with methanol/acetone/DMSO (2 : 1 : 1 by vol.), non-radioactive BP meta-
bolite standards added, and the extract chromatographed on a Dupont
Instruments Model 848 High Pressure Liquid Chromatograph with 4 mm
× 30 cm μ-Bondapak C$_{18}$ column (Waters Associates) using an isocratic
elution solvent of methanol/water/ethyl ether (66.3 : 30.4 : 3.3, by vol.)
at a flow rate of 1.4 ml/min. The effluent was monitored by UV spectro-
metry to identify metabolites, which were quantitated by collecting appro-
priate fractions of the effluent for liquid scintillation analysis. Six second
fractions were collected for 11—12 min, then 12-s fractions were collected
for 8—9 min and lastly, 60-s fractions were collected until the completion
of the chromatographic run. Typical retention times in minutes for each
metabolite were: BP-9,10-diol, 3.6; BP-4,5-diol, 5.5; BP-11,12-diol, 5.8;
BP-7,8-diol, 6.5; BP-1,6-quinone, 8.6; BP-11,12-quinone, 9.1; BP-3,6-qui-
none, 9.6; BP-6,12-quinone, 11.0; BP-9-phenol, 16.5; BP-3-phenol, 19.1;
BP, 37.5. The overall recovery of radioactivity from the column was greater
than 90%.

RESULTS

[G-^{3}H]BP-protein complexes isolated from the cytoplasm of low and
high passage cells were chromatographed on a Sephadex G-200 column.
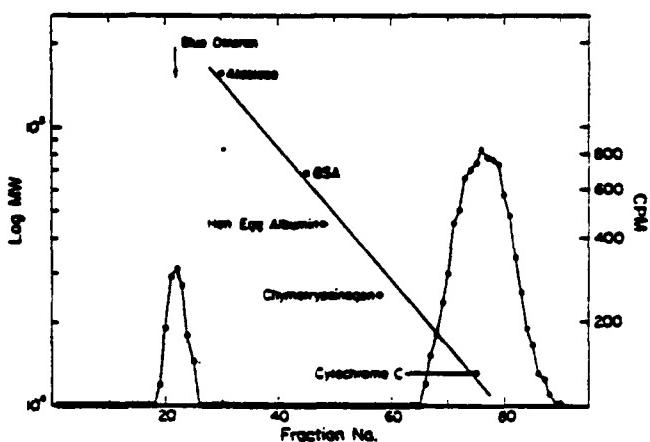
Fig. 1. Sephadex G-200 chromatography of a [G-³H]BP-cytoplasmic protein complex from low passage cells. The cytoplasmic fraction was prepared from passage 5 HNF cells treated with [G-³H]BP for 12 h. 50,000 dpm were applied to a Sephadex G-200 column (58 × 0.9 cm) and eluted with 0.01 M Na phosphate — 0.3 M Tris—HCl — 2.5 mM Na₂EDTA — 5 mM DTT (pH 7.5). 0.5-ml fractions were collected and the radioactivity was assayed. Blue Dextran — 200,000; Aldolase — 158,000; Bovine Serum Albumin — 67,000; Hen Egg Albumin — 45,000; Chymotrypsinogen — 25,000; and Cytochrome c — 12,500 served as molecular weight standards.
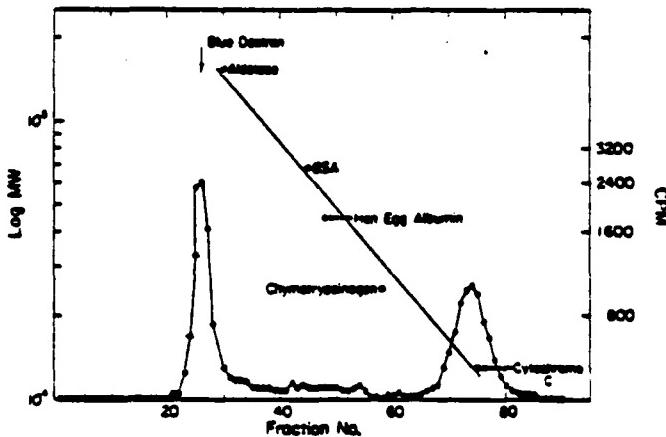

Fig. 2. Sephadex G-200 chromatography of a [G-³H]BP-cytoplasmic protein complex from high passage cells. The cytoplasmic fraction was prepared from passage 25 HNF cells treated with [G-³H]BP for 12 h. 100,000 dpm were applied to a Sephadex G-200 column (58 × 0.9 cm) and 0.5 ml fractions were collected as described under Fig. 1.

In low passage cells, a major portion of BP was associated with a protein complex of molecular weight 12,500. The ratio of area under the low molecular weight peak to the high molecular weight peak was 6.5 (Fig. 1). In high passage cells, a major portion of BP was associated with a protein complex of molecular weight 200,000 and the ratio of the area under the
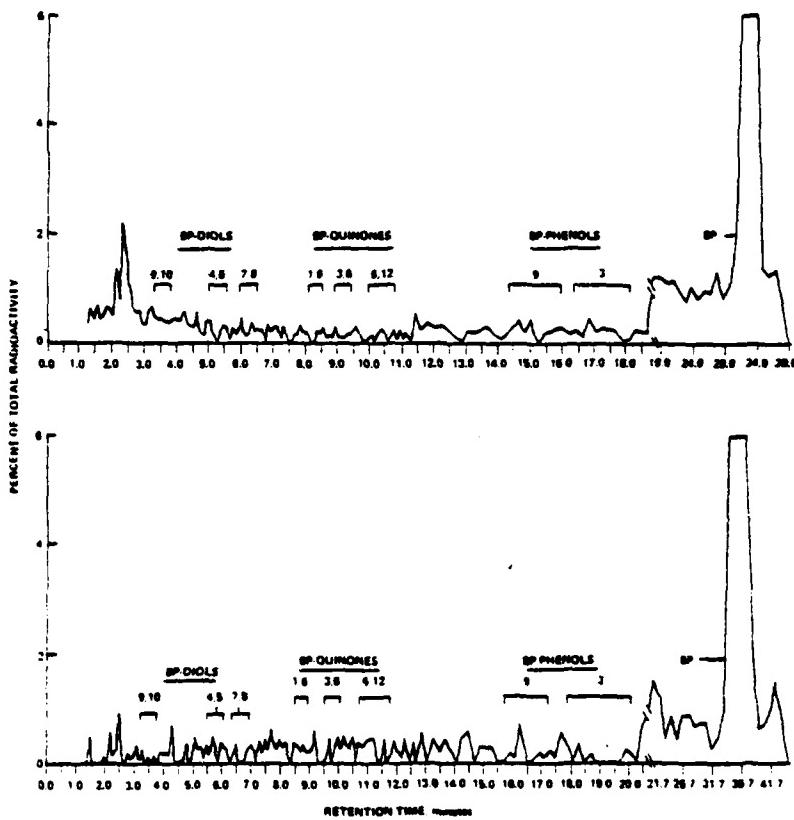
Fig. 3. HPLC profiles of the ethyl acetate extractable radioactivity from the total cytoplasmic protein-hydrocarbon complexes of low and high passage cells. Non-radioactive BP metabolite standards were cochromatographed with the radioactive extract for metabolite identification. An isocratic elution solvent of methanol/water/ethyl ether (66.3 : 30.4 : 3.3, by vol.) was employed at a flow rate of 1.4 ml/min and fractions collected for liquid scintillation spectrometry. (See Material and Methods for details.) Upper panel: Profile of the organic extract of [G-³H]BP-total cytoplasmic protein complex from high passage human diploid fibroblasts. A total of 6300 dpm were applied to the column. Lower panel: profile of the organic extract of [G-³H]BP-cytoplasmic protein complex isolated from low passage human diploid fibroblasts. A total of 4000 dpm were applied to the column. Note the discontinuity in the retention time scale.

high molecular weight peak to the low molecular weight peak was 1.8 (Fig. 2).

The total cytoplasmic protein complex isolated from low passage human fibroblast cells exposed to [G-³H]BP was extracted with ethyl acetate, and the non-covalently bound hydrocarbon and its metabolites were co-chromatographed on a reverse phase column by high-pressure liquid chromatography with authentic reference standards. In the metabolite profiles of
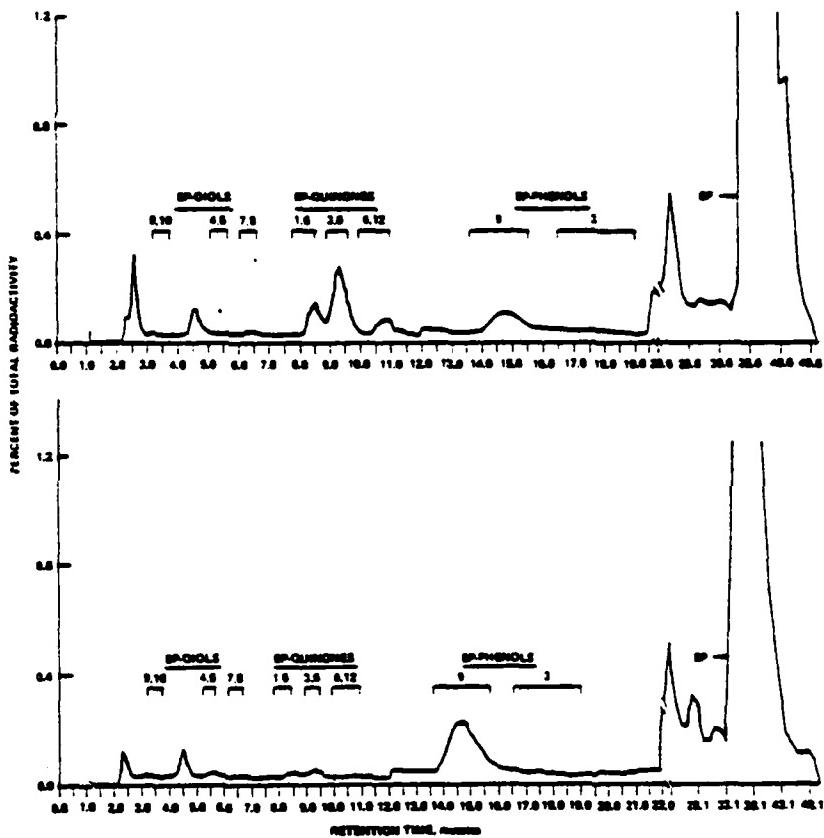
Fig. 4. HPLC profiles of the ethyl acetate extractable radioactivity from nuclei of low and high passage human diploid fibroblasts exposed to [G-³H]BP. Non-radioactive BP metabolite standards were cochromatographed with the radioactive extract for metabolite identification. (See Fig. 3, Materials and Methods for details). Upper panel: profile of the organic extract from nuclei of high passage cells. A total of 1.263×10^{6} dpm were applied to the column. Lower panel: Profile of the organic extract from nuclei of low passage cells. A total of 560,850 dpm were applied to the column. BP-11,12-diol and BP-11,12-quinone cochromatographed with BP-4,5-diol and BP-3,6-quinone, respectively.

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BP-treated low passage cells (Fig. 3), unmetabolized BP was the only radio-labeled fraction. Similar results were obtained with BP radioactivity isolated from the total cytoplasmic protein complex of high passage cells, except that a small peak of radioactivity eluted prior to the BP-9, 10-diol (Fig. 3).

The radioactivity associated with isolated nuclei of low and high passage cells treated with [G-³H]BP was chromatographed as described above (Fig. 4). The nuclei from high passage cells had radioactivity which co-chromatographed with BP-1, 6; 3,6 and 6,12-quinones and BP-9-phenol; 89% of the counts were associated with BP; however, the radioactivity isolated from [G-³H]BP-treated low passage cells co-chromatographed with BP-9-phenol, with 90% of the counts eluting with BP. The unknown peaks at 2.4 and 4.5 min represent void volume radioactivity (pre-BP-9,10-diol) and an unidentified metabolite, respectively. Co-chromatography with BP-11,12-diol and BP-11,12-quinone indicated that the 4.5 min peak represented neither of these potential metabolites.

DISCUSSION

When low passage HNF cells are treated with BP, the PNH accumulates in the cytoplasm, before localizing in the nucleus 24 h after exposure. Sephadex G-200 gel chromatographic separation of the cytoplasmic protein complexes from low passage and high passage cells indicated the distribution of BP between protein complexes of molecular weights, 12,500 and 200,000. In low passage cells, the amount of BP associated with the low molecular weight protein complex was 4--7 times that associated with the high molecular weight protein complex. However, in high passage cells, the amount of BP associated with the high molecular weight protein complex was 0.8--1.8 times that associated with the low molecular weight protein complex. HPLC analysis of the BP radioactivity separated from the BP-total cytoplasmic protein complex of low passage and high passage cells indicated that the major fraction was the parent BP. This is in contrast to a previous study [10], in which active metabolites of 3'-methyl-4-dimethyl amino azobenzene were shown to bind to a cytosol protein complex from rat liver. It was also interesting to observe that unmetabolized [G-³H]BP made up the major fraction of the PNH associated with the nuclei from low passage and high passage cells. The minor peaks observed in the HPLC metabolite profiles may be a result of autoxidation of the sample, although all procedures were performed under red light, argon, and in the presence of an antioxidant.

We have observed minor differences in the metabolites non-covalently bound to DNA, in low and high passage cells. Also, as described above, we have observed binding of BP to different cytoplasmic protein complexes in low and high passage cells. The transport of BP into the nucleus of these cells may involve an activation of the BP-cytoplasmic protein complex, similar to that observed with the steroids. Only binding of BP

to the lower molecular weight protein complex (the predominant complex in low passage cells) may result in an activation of the complex. Therefore, the accessability of BP metabolites to specific nuclear binding sites in low and high passage cells may be different and may account for the susceptibility or refractoriness to BP-induced carcinogenesis of human fibroblast cells in vitro.

ACKNOWLEDGEMENT

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$\overline{IV}-2$

GROWTH AND ULTRASTRUCTURAL CHARACTERIZATION
OF PROLIFERATING HUMAN KERATINOCYTES IN VITRO
WITHOUT ADDED EXTRINSIC FACTORS[1]

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SUMMARY

Routine in vitro cultivation of human epithelial cells derived from foreskin and free of
contaminating fibroblasts has been achieved without the addition of conditioned medium
or extrinsic factors. Epithelial cell populations could be serially subpassaged and exhibited
modulating responses at PDL5 to culture conditions as the cells passed from phase 1
through phase 2 of their life span. The cell population in early phase 2 gave rise to tissue
sheets that exhibited characteristics typical of human foreskin epidermis including the
formation of distinct cellular layers. viz. strata basalis. spinosum. granulosum and
corneum. Typical keratohyaline granules were not observed in the epithelial cells although
a distinct cornified layer was evident. Ultrastructurally, desmosomes and tonofilaments
were readily apparent. Thus. the procedure detailed in this study will produce highly dif-
ferentiated fibroblast-free epidermal sheets reaching several centimeters in size and which
can be removed from the substratum as a single sheet of organized epidermis. The epi-
thelial cells could be cultured through 20 ± 3 PDL. whereas fibroblast cultures derived
from foreskin cultures exhibited 40 ± 5 PDL and mixed cell cultures of foreskin were car-
ried through 43 ± 5 PDL.

Key words: epithelial cells; human skin cultures; skin epithelial cells; epidermis culture;
ultrastructure of cultured epithelial cells.

INTRODUCTION

Reproducible in vitro cultivation of normal pro-
liferating human epithelial cells has been difficult
to achieve with present methodologies and the
procedures developed to establish primary epi-
thelial cell populations have resulted in a low rate
of success (1–4). Enhanced establishment of epi-
thelial cell populations from explants (5,6) occurs
with the addition of fibroblasts or extrinsic growth
factors, i.e. products released from cultured fibro-
blasts (conditioned growth media) (7), epidermal
growth factor (3) or hydrocortisone (8), to the cul-
tures or culture media. Recently, Freeman et al.
(9) reported that by the use of a dermal collagen
bed derived from sterile pig skin. human epi-
thelial cell cultures were established in 129 of 140
attempts; the cultured epithelial cells grew in the
absence of fibroblasts or their products (except
collagen). Earlier. we described a method for the
enzymatic dispersion. growth and serial subpas-
sage of primary cultures of human fibroblasts de-
rived from foreskin (10); epithelial colonies were
occasionally noted in these cultures after subpas-
sage. We have now modified the culture technique
in a manner that permits not only the establish-
ment of pure populations of human fibroblasts
but. more importantly. allows for the successful
establishment of pure human epidermal epithelial
cell cultures. The human epithelial cells were
grown successfully without the addition of extrin-
sic growth factors or collagen substrata and have

been serially subpassed. fibroblastfree. on a routine basis. Epithelial cells could be grown to form large multilayered epidermal-like sheets in which the cells showed differentiative characteristics consistent with those noted in normal intact epidermis for a limited number of PDL: (PDL 5).

It is the purpose of this report to detail the methodology used for the routine culture of human foreskin epithelial cells (keratinocytes), which grow to form large multilayer cell sheets. The growth characteristics and morphology of the cultured cells also will be described.

MATERIALS AND METHODS

Preparation of cell suspensions. Human foreskin was obtained from infants at the time of circumcision. The epidermis was dissected carefully from the underlying connective tissue dermis and cut into 2-mm pieces in MEM-Hanks' balanced salt medium containing 25 mM HEPES buffer at pH 7.2 ((CM) culture medium]. The tissue was rinsed three times in this medium and the tissue fragments transferred to 20 ml of CM medium supplemented with 20% fetal bovine serum (FBS) containing 0.25% collagenase (115 U per mg, 4197 CLS, Worthington Biochemical Corp.. Freehold, New Jersey). Enzymatic tissue dispersion was done at 37° C in a 4% CO₂-enriched air atmosphere for 5 hr or overnight. Cells were recovered from suspension by centrifugation at $150 \times g$ for 7 min at 4° C. The cell pellet was washed twice with CM medium and seeded into 75-cm³ flasks. After seeding, the cell cultures had to be refed at 48 hr with 15 ml of the CM medium supplemented with 20% FBS. Three to five days later. cultures were observed for the appearance of epithelial colonies. and the mixed cell cultures were allowed to grow to confluency. It should be noted that epithelial cell growth was dramatically inhibited by addition of either penicillin, streptomycin. aureomycin or fungizone. Therefore, antibiotics were not added to the culture medium.

Preparation of epithelial cell cultures. At confluent density or when cultures reached a diameter of 5 to 9 mm, primary mixed cell cultures were trypsinized in order to remove fibroblasts. The longer the cultures were left in confluent density, the more difficult it became to selectively remove the fibroblast population: 16 hr after the cultures reached confluency proved an optimum time to do this. CM was removed from the mixed cell cultures and the cultures rinsed twice. One milliliter 0.1% trypsin (Worthington Biochemical Corp..

lypholized 9300 BAEE U per mg, lot TL-3BP) in CM was layered over the culture monolayers.

After 90 seconds. the fibroblasts floated off the substratum while the epithelial sheet remained firmly attached to the substratum. The enzymatic action was stopped by the addition of 10% FBS-supplemented CM. Residual fibroblasts were removed by rinsing the flask twice with growth medium. These fibroblasts were seeded in separate culture vessels and subpassed as previously described (10). The epithelial cultures were fed with 11.5 ml CM medium. 1.5 ml FBS and 3 ml minimum essential vitamin mixture (100x concentrated) [CM plus vitamin supplemented (CM-V). Microbiological Associates. Walkersville, Maryland]. The reduction in supplementation of FBS from 20 to 10% or even 5% diminishes the growth rate of residual fibroblasts while not adversely affecting the growth of the epithelial cells. Trypsinization was repeated 2 to 4 times at 3-day intervals. Epithelial cultures were allowed to grow for at least 2 weeks and were refed every 4 days with CM-V.

Serial subpassage of epithelial cells. Subpassage was initiated within 2 to 4 weeks after seeding of the primary cultures. In preparation for subpassage. the CM-V medium was decanted and the epithelial cell sheet rinsed with 10 ml of Mg^{+1}-, Ca^{+1}-free MEM containing 0.02% tetra sodium ethylene diaminetetraacetate (EDTA; Eastman Kodak. Rochester. New York) at pH 7.2. Cultures were then treated with a 0.1% trypsin solution in Mg^{+1}- Ca^{+1}-free MEM. 0.02% EDTA. for 90 seconds. Trypsin activity was neutralized by addition of 15 ml of CM-V medium containing 10% FBS. The epithelial colonies lost continuity and individual cells became detached from the substratum. The dish (or flask) was gently shaken increasing detachment; free cells floated in the medium.

The free-floating epithelial cells were recovered by centrifugation at $150 \times g$ for 7 min, and the cell pellet was resuspended in CM-V medium supplemented with 10% FBS. After rinsing the pellet once in CM-V medium. the suspended cells were seeded into 25-cm³ flasks or plates at a cell density of 50,000 cells per cm³. Subsequent subpassages were done in a similar manner. Within 15 to 20 min after seeding, the cultures were gently rotated by hand for about 5 min to encourage aggregation of the single cells before attachment to the substratum: without this step a noticeable decrease in the numbers of subsequent epithelial colonies was observed.
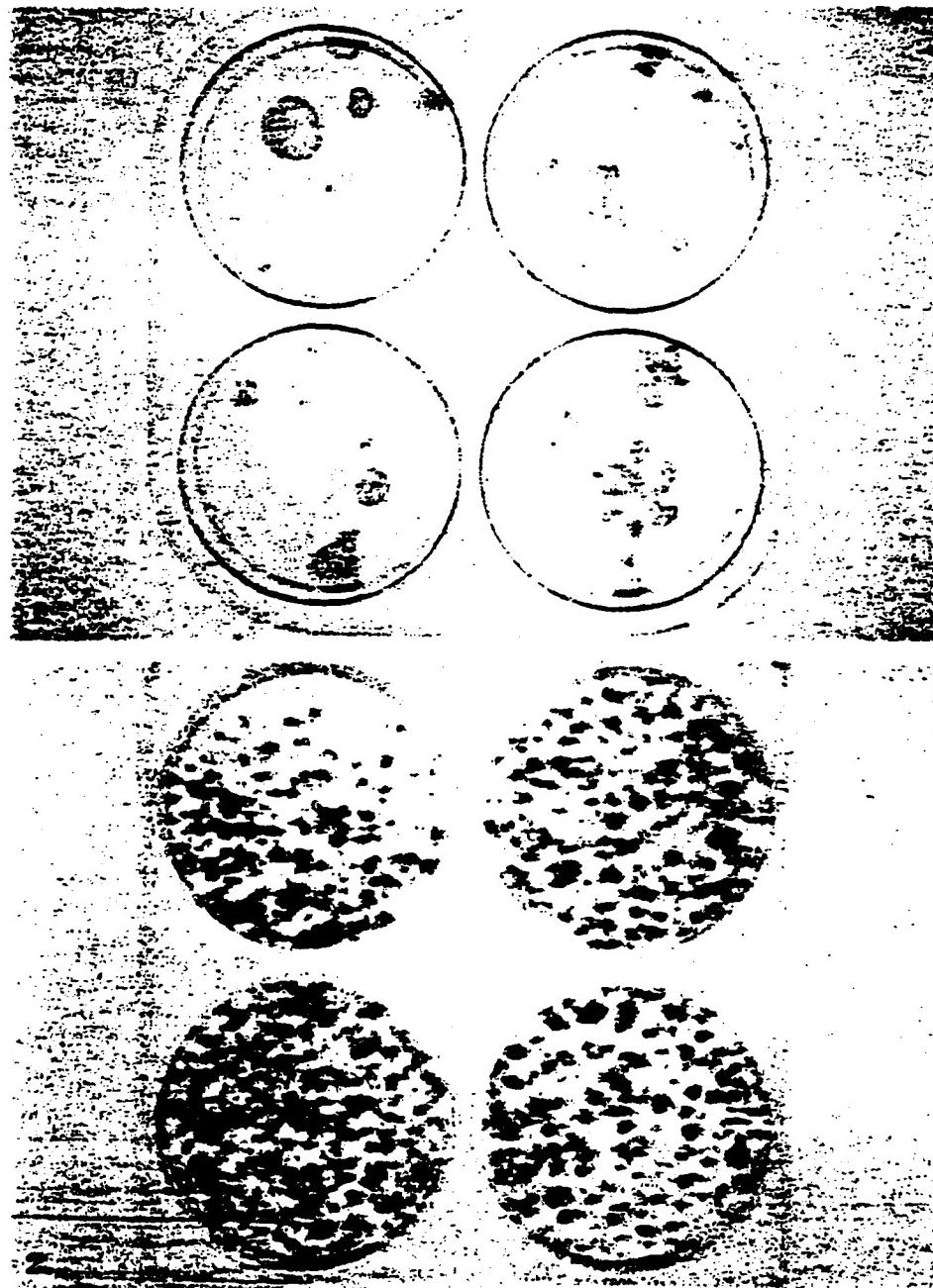
FIG. 1. Colonies of epithelial cells after 3 weeks after seeding 500 cells per dish (25 cm²), buffered with formalin and stained with hematoxylin eosin. ×½.

FIG. 2. Colonies of human fibroblast seeded at 1000 cells per dish (25 cm²) from PDL 2, fixed in phosphate buffered formalin and stained with hematoxylin eosin. ×½.

TABLE 1

GROWTH CHARACTERISTICS OF FIBROBLAST. EPITHELIAL AND MIXED CELL POPULATIONS IN VITRO

Culture Type	Cells seeded (75 cm²)	Cell Density at 7 Days (75 cm²)	PDL (Days) After 1:4 Splits	Life Span PDL
Fibroblast	375,000	$1.5 - 2 \times 10^6$	3	40 ± 5
Epithelial[a]	250,000	$4 - 6 \times 10^6$	14	20 ± 3
Mixed	25,000	2×10^6	3	43 ± 5

[a] These cell populations exhibit alterations in morphology as they are serially subpassaged.

Preparation of epithelial and fibroblast cultures for microscopy. Cell cultures examined for their growth patterns and morphology in culture were fixed in formalin and stained with hematoxylin. For electron microscopy, epithelial cultures were washed with CM media and exposed to 0.1% collagenase in 10% FBS-supplemented growth medium at 37° C in 4% CO_2-enriched air atmosphere for 4 to 12 hr in order to free the cells or colonies from the substratum. These cell sheets (2 to 50 cm² in area) were removed and fixed in 3% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.4, for 30 min at room temperature or overnight at 4° C. Confluent cultures of fibroblasts were scraped into sheets and fixed in 3% glutaraldehyde. Cell preparations were subsequently postosmicated in 1% chrome-osmium tetroxide for 1 hr at 4° C, dehydrated and embedded in Araldite. Thick (1 μm) sections were stained with 1% basic fuchsin in 50% acetone or with aqueous 0.1% toluidine blue for light microscopy. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

RESULTS

Growth characteristics of epithelial and fibroblast populations. Epithelial cultures, freed of fibroblasts, grew in discrete colonies (Fig. 1). Cell colonies at confluency formed large continuous sheets (75 cm²) in size. On occasion, the cell sheet would spread up the side of the well or around the neck of the culture flask, a feature never observed with fibroblast cultures. Optimal cell density for seeding of epithelial cultures was found to be 50,000 cells per cm² and a population doubling occurred after 14 days (11). Pure epithelial cultures (PDL 2 to 5) exhibited cellular stratification rather than forming true monolayers: cells were always found to be in contact or intimately attached with the adjacent cells of the patch or

FIG. 3. Transverse section through the more central region of an epithelial sheet from cultures at PDL 1. Note stratification of the epithelial cells: stratum basalis. B: stratum spinosum. S: stratum granulosum. G: stratum corneum. C. 1-μm Araldite section stained with basic fuchsin. ×700.

FIG. 4. Transverse section through the marginal region of the epithelial sheet from cultures at PDL 1. Note that the number of cell layers and thickness of this area is less than in Fig. 1. Individual strata can be seen. Stratum basalis. B: stratum spinosum. S: stratum granulosum. G: stratum corneum. C. 1-μm Araldite section stained with basic fuchsin. ×700.

sheet. These growth characteristics were maintained for five PDL after which distinctive changes occurred in the growth pattern that will be detailed in a subsequent paper. Fibroblast populations removed from the mixed foreskin cultures and seeded on a separate substratum exhibited typical fibroblast growth patterns (Fig. 2). growing in definite parallel whorl-like patterns. which were of variable size: cells were not attached to one another. The fibroblast growth characteristics were maintained for PDL 40 (Table 1) when cloned from 100 cells per cm² or at a high density of 5.000 cells per cm². Saturation growth density of subcultures of fibroblasts decreased from 20.000 cells per cm² (PDL 1 to 15) (early phase 2) to 15.000 cells per cm² (PDL 16 to 31) (middle phase 2). and cultures would not reach a confluent state after PDL 32 (Table 1) (late phase 2).

Microscopy of the epithelial cell population. Light microscopic examination of the epithelial colonies demonstrated their stratified nature (PDL 1 to 5). The central region of the colonies was 6 to 8 cells in thickness (Fig. 3). whereas the marginal zone was much thinner and consisted of 3 to 5 cell layers (Fig. 4).

FIG. 5. Epithelial cells of the basal (B) and spinosum (S) layers. Note filament (F) bundles and desmosomes (D). Several mitochondria (M) are also indicated. Stained with uranyl acetate and lead citrate. ×27.000.

(Fig. 9). Typical keratohyaline granules were not observed in the epidermal cells although present in the native foreskin epidermis. Relatively few mitochondria were present in the epithelial cells of the stratum granulosum and nuclei were uncommon in this layer of the cultured epithelial sheets. Small electron-dense, round-to-oval, membrane-bound granules with electron lucent clefts or zones (Figs. 6,7) provided an additional morphological feature of the granular cells and cells of the stratum spinosum in immediate apposition to the stratum granulosum. It is noteworthy that these small granules concentrated along the plasma membrane on the side of the cell directed toward the stratum corneum. These small granules closely resemble morphologically, and by posi-

tion, the mucus-coating granules (MCG) described in normal epidermis.

The most superficial layer of the epidermal sheet (Figs. 7, 10) resembled the typical stratum corneum of foreskin epidermis, although this stratum was only one to two cells thick in the culture preparations. The cells were quite flattened and lacked nuclei and cell organelles. Tonofilaments were abundant, oriented parallel to the long axis of the cell and embedded in an amorphous material of low electron density (Fig. 10). The plasma membrane was thickened when compared to the cell membranes of other cells of the epidermal sheet. Modified desmosomes, similar to the modified desmosomes described for normal epidermis, were evident between the cornified

FIG. 7. Superficial portion of the epithelial sheet shows a cornified cell (C) and several cells of the stratum granulosum. Secondary lysosomes or lipofuchsin granules (L) are evident. In addition, note the small granules (O) concentrating near the plasma membrane. Stained with uranyl acetate and lead citrate. ×21,000.
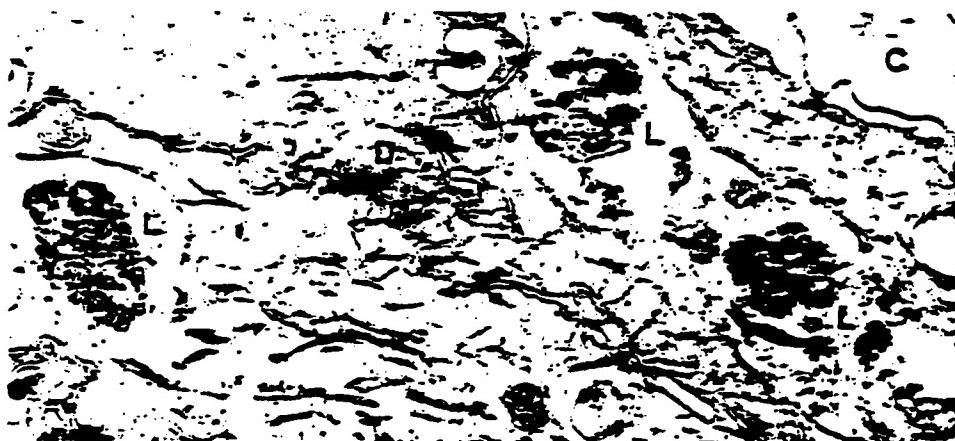
FIG. 8. Higher magnification of the secondary lysosomes or lipofuschsin granules (L) of granular cell in the stratum granulosum demonstrates their variable internal structure. Desmosomes are shown at D and a portion of an epithelial cell (C) of the stratum corneum. Stained with uranyl acetate and lead citrate. ×27,000.

(keratinized) cells. Transitional forms between typical granulosal cells and the superficial cornified cells were found.

Morphology of the fibroblast population. Fibroblast cultures derived from the mixed cul-

tures of foreskin appeared quite distinct (Fig. 11) from the cells of the epithelial colonies and sheets. Most of these cells occurred singly. Cells in contact with one another were not attached by desmosomes. Fibroblasts contained both smooth and

FIG. 9. Epithelial cell located at the junction of the stratum spinosum and granulosum. Note morphological stages in the formation of the large secondary lysosomes or lipofuscin granules (L). Cytoplasmic filaments (F) and mitochondria (M) are indicated. Stained with uranyl acetate and lead citrate. ×44,000.

rough endoplasmic reticulum. The rough endo-
plasmic reticulum was frequently distended and
such profiles had few attached ribosomes. Mito-
chondria were numerous and glycogen was abun-
dant; autophagic vacuoles were present. At higher
magnifications, cytoplasmic filaments were evi-
dent but were less numerous than in the epithelial
cell and did not form discrete bundles as seen in
the epithelial cell cultures.

DISCUSSION

We have been able to culture and serially sub-
pass epithelial cells derived from normal and
human foreskin. Pure epithelial cultures free of
contaminating fibroblasts were obtained and
maintained in typical epithelial-like cultures
through five PDL. Epithelial growth patterns
were distinctive, and cell colonies, when grown to
confluency, formed large sheets several layers in
thickness with adjacent cells joined by desmo-
somal junctions. Self-limiting islands of epithelial
cells surrounded by fibroblasts, noted by others

using alternative procedures to prepare epithelial
cell cultures in vitro (7.9.12–15), were not ob-
served in this study. Cells comprising the epi-
thelial sheets exhibited differentiative changes
identical to those occurring in normal epidermis
of the intact foreskin. Distinctive cell strata were
observed in the epithelial cultures. Epithelial cells
or keratinocytes possessed tonofilaments, desmo-
somal junctions and mucus-coating granules.
Thickened cell membranes of the enucleated
superficial cornified cells plus modified desmo-
somes and organized tonofilaments in an amor-
phous matrix characterized these fully differenti-
ated surface epithelial cells (19). Differentiative
changes in each cell strata of the cell sheets were
identical with those seen in normal epidermis ex-
cept that keratohyaline granules (20) were absent
in the stratum granulosum; however, secondary
lysosomes or lipofuchsin granules were a con-
spicuous feature of the cells of the stratum granu-
losum of the epithelial cultures. The absence of
keratohyaline granules may reflect the cell's in-
ability to synthesize these granules while rapidly
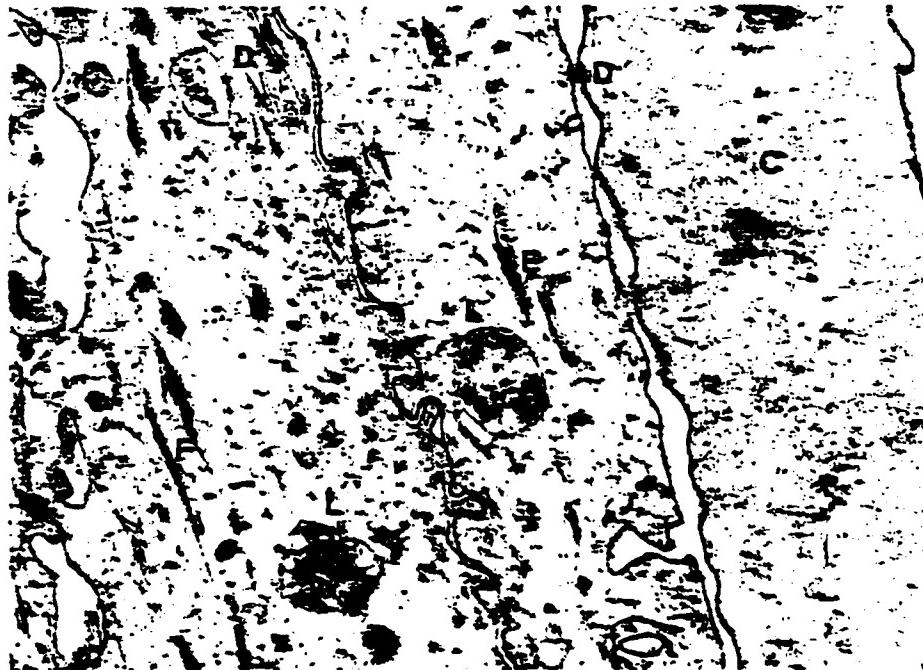

FIG. 10. Note the tonofilaments (F) have assumed an orientation along the long axis of the cells of the
stratum granulosum. Desmosomes (D) and secondary lysosomes (L) are evident. The plasma mem-
brane of cornified cell (C) is thickened and fine filaments are embedded in an amorphous matrix in this
superficial cell of the epithelial sheet. Stained with uranyl acetate and lead citrate. ×29.000.
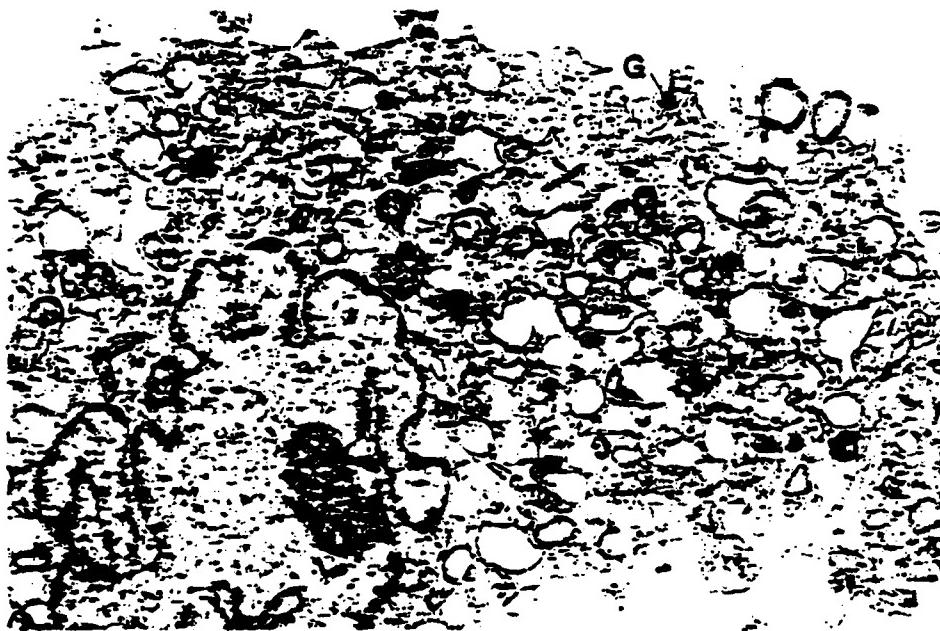
FIG. 11. Cultured fibroblasts derived from the mixed cultures are morphologically distinct from the cells of the epithelial sheets. There are no desmosomes and few filaments can be distinguished. Mitochondria are numerous, scattered dilated cisternae are evident and a number of small vacuoles are present in the cytoplasm; glycogen (G) is a common feature of these cells. Stained with uranyl acetate and lead citrate. ×17,000.

proliferating or because of in vitro culture conditions.

Variable degrees of success have been reported in the propagation of normal human epithelial cells, particularly those from human epidermis or skin. Explant culture of human epidermis commonly exhibited fibroblastic growth in association with the epithelial outgrowths suggesting that fibroblast interaction was necessary for achieving epithelial propagation and differentiation (16). In the system described here the presence of fibroblasts does not enhance growth of the epithelial cell population. On the contrary, fibroblasts, when left in mixed cell cultures, overgrow the epithelial cells, thereby inhibiting epithelial growth. After contaminating fibroblasts from mixed primary cultures of human foreskin were removed by selective trypsinization, we were able to subpassage the pure epithelial cell population up to five PDL without morphological alteration. Others (17) were able to subpass normal adult skin through four subpassages with modest differentiation evident in the culture strata.

Growth of human cell populations can be affected by the composition of the fetal bovine serum (15). Traditionally, we characterize (18) the fetal bovine serum prior to use on human cell populations. In addition, we have found that several types of antibiotics inhibited the establishment of proliferating epithelial cell cultures. We have found that the growth characteristics, cell attachments, proliferative characteristics and life span of the cultured epithelial cells from human foreskin were finite and were markedly different from cell cultures arising from a mixed cell population. Fibroblast cultures, derived from infant foreskin subcultures, when grown to confluency, ceased to grow except at terminal points of the whorling patterns; overlapping of cells only occurred at these sites. In contrast, epithelial cells grew in concentric ring patterns and were several cells in thickness.

Preliminary comparison of growth characteristics, as noted in Table 1, indicated that the life spans of epithelial cell populations were different in extent of proliferation from fibroblast PDL.

Moreover, the epithelial cell populations did exhibit characteristic senescent features in vitro. These cell populations, like the fibroblasts, passed through phases 1 and 2 (11) as seen by Karasek and Liu (19). They did not exhibit saturation density-dependent inhibition. The piling up of the epithelial cells into strata (20.21) may account for the increase in numbers of cells observed in the epithelial cultures. We have found that foreskins from adults can produce a ready source of keratinocyte cells that can be grown in vitro using the method described here. These epithelial cell populations also exhibit the growth characteristics associated with tissue in phase 2 and differentiative structures similar in anatomical characteristics to skin epidermis. This differentiative tissue produced in vitro does not require added extrinsic factors, such as epidermal growth factor, pituitary extract, conditional growth medium, hydrocortisone or the presence of collagen to restrict the proliferation of fibroblasts.

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ESTABLISHMENT OF PROLIFERATING HUMAN EPITHELIAL CELLS IN VITRO FROM CELL SUSPENSIONS OF NEONATAL FORESKIN

Submitted by

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I. INTRODUCTION

The following procedure has been successfully applied to many different human tissues. Tissue samples are obtained from cooperating hospitals. Using the collection techniques described here, we can retain excellent viability from 96 hr up to 5 days (postcollection) depending on the tissue of choice and source of tissues. The use of this procedure permits the establishment of epithelial cell cultures from cell suspensions not requiring explant growth or the addition of extrinsic modulating growth factors. Moreover, epithelial cell colonies can be produced at a low density or high density directly from the cell suspension.

Key words: primary; epithelial cells; cell suspensions.

II. MATERIALS

Minimum essential medium (MEM) Eagle with Hanks' salts (HBSS) (GIBCO[1]) and 25 mM HEPES; without glutamine and $NaHCO_3$. To 100 ml of the medium, add 1 ml non-essential amino-acid mixture (10 mM, Micro[2]), 1 ml sodium pyruvate (100 mM solution[2]), 0.1 ml Gentocin (50 mg per ml, Schering[3]), 1 ml L-glutamine (200 mM[2]), and titrate with 8.8% $NaHCO_3$ solution (sterile, carbonate-free) to pH 7.2. Designated complete growth medium (CM).

MEM Eagle without magnesium and calcium[2] (not supplemented unless mentioned in Procedure section), for suspension (spinner) cultures. Designated spinner medium (SM).

Dulbecco's LoCal medium. Biolabs.[4] Supplement exactly as CM. Designated LoCal.

Trypsin, lyophilized. No. TL 13 BP Worthington[5] (1% solution made up in MEM Eagle HBSS medium)

Essential vitamin mixture, 100X[2]

Collagenase (CLS), No. 4197.[5] Suspend 1 g collagenase in 100 ml MEM Eagle HBSS, pH 7.2; dissolve with magnetic stirrer at 4° C; centrifuge 10.000 × g at 12° C for 10 min; and filter through a 0.22-μm filter (No. 7103 Falcon[6]).

Fetal bovine serum (FBS)[7] [evaluated for steroid composition (1), unsaturated fatty acid composition (2), mycoplasmal contamination, etc. (3), and growth properties on indicator cells (4)]

Stirring bars, Teflon-molded, magnetic, ⅞-inch long, ⁵⁄₁₆-inch diameter, No. 6006 Bellco[8]

Culture plates, four wells per plate, 28-cm², No. 3004[6] or No. FB-4-TC Linbro[9]

Scalpels, disposable, sterile, No. 32 390-0222 AHS[10]

Tissue culture flasks: 75-cm², No. 3024[6] or No. 5375[6]; or 25-cm², No. 25100 Corning[11]

Plastic pipettes, measuring (Mohr), plugged: 5-ml. No. 7532; 10-ml No. 7548[6]

Centrifuge tubes, conical, plastic, 15-ml. No. 3013-000[11]

Ethylenediamine tetracetate (EDTA), tetrasodium salt[12]

III. PROCEDURE

A. Collection of human tissue

1. Supply the operating room with several 4-oz bottles containing 10 ml CM at pH 7.2 supplemented with 5% FBS and 0.1 ml Gentocin per 100 ml medium. These bottles may be stored at 12° C for many

weeks (in our hands, 3 to 4 weeks, depending on composition of the glass). If there is a drastic change in pH, discard the bottles, i.e. if the pH rises above 7.2 (color shifts from red to purple) or falls below 6.8 (color shifts from red to yellow).

2. Collect tissue on the average of 3 or 4 times a week. We have found that when the tissue is kept in CM plus 5% FBS at 12° C, 95% viability is retained up to 48 hr after collection.

B. Processing of tissue

1. Charge each of four wells of a 28-cm² culture plate with 5 ml CM.

2. Place the tissue from the collection bottles into the first well, swirling the medium to wash the tissue.

3. Transfer the tissue to the second well and wash.

4. In the third well, cut the tissue into three or four segments and wash.

5. Transfer segments to the fourth well and mince with two scalpels into 2-mm pieces.

   a. Swirl the medium to rinse the tissue.

   b. Suck off the medium with a narrow-mouth pipette leaving only the minced tissue.

6. Defrost a 5-ml vial of 1% collagenase and add to the well containing the minced pieces. Have ready a 75-cm² flask containing 15 ml CM supplemented with 20% FBS. Transfer the minced tissue and collagenase to the preincubated flask, thereby diluting collagenase to 0.25%.

7. Incubate the tissue at 37° C in a 4% CO₂ environment overnight (16 hr). For a period of 5 to 7 hr of incubation, use 0.5% collagenase.

8. Transfer the digest into a 15-ml plastic or glass conical centrifuge tube. (Use plastic pipette with a 1.5-mm diameter aperture.)

9. Centrifuge the sample for 7 min at $650 \times g$ at 4° to 12° C.

10. Resuspend the pellet in 5 to 10 ml CM supplemented with 20% FBS; recentrifuge again as described in step 9.

11. Repeat step 10.

12. Preincubate a 75-cm² flask containing 10 ml CM supplemented with 20% FBS for 30 to 45 min at 37° C in a 4% CO₂ environment.

13. Suspend the pellet obtained from step 11 in 5 ml CM at 20% FBS. Seed one 75-cm²

flask or three or four 25-cm² flasks with the cell suspension.

14. Incubate the flasks at 37° C in a 4% CO₂ environment.

15. Two days later, rinse the primary culture with CM and refeed with CM supplemented with 20% FBS.

16. Three to five days postseeding, check for epithelial colonies.

17. Fibroblasts also will be present in these cultures. Three to five days after seeding, when epithelial colonies are well established, selectively trypsinize the cultures to remove the fibroblasts. PRECAUTIONARY NOTE: This step is a critical procedure and particular attention must be paid to it. The epithelial islands should be left relatively undisturbed.

18. Decant growth medium from the mix cultures and rinse with 10 ml CM.

19. At this time, remove from the freezer the trypsin prepared as a 1% solution in CM at pH 7.2.

    a. Dilute with CM to 0.1%.

    b. Add 1 ml 0.1% trypsin to the cell sheet.

    c. Incubate at 21° C (room temperature) for approximately 30 sec.

    d. Observe the cell sheet under 10X magnification to determine when fibroblasts lift off the substratum. The epithelial patches will remain attached to the flask.

20. To stop the action of trypsin, add 10 ml CM supplemented with 20% FBS and use this medium to wash the cell sheet to remove fibroblasts.

21. Decant medium and repeat step 20.

22. Do not attempt to remove all fibroblasts during this first trypsinization. When the edges of the epithelial patches begin to retract, immediately stop trypsin action. It is better to repeat steps 18–20 the next day than to continue to remove all the fibroblasts at this time.

23. Refeed the flasks containing mainly epithelial colonies with CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium. The lowered FBS supplementation retards the growth of any remaining fibroblasts while the vitamin supplementation encourages epithelial growth.

24. Repeat trypsinization procedures two to four times at approximately 3-day inter-

vals or as necessary to free cultures from fibroblasts.

C. Subpassaging epithelial cells

1. Place epithelial cells for 2 to 3 days on LoCal containing additives and supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml of medium. Pretreatment with calcium-deficient medium greatly facilitates the lifting off of the epithelial cells from the substratum during trypsinization.

2. Decant the LoCal medium, rinse the cell sheet with 10 ml SM containing 0.02% EDTA, and incubate at 37° C for 3 to 5 min. During this time epithelial cells will begin to separate along their boundaries but will continue to remain attached to the substratum. An increase in refractoriness along the outer limits of each cell will be noticed.

3. Decant the SM and add 1 ml 0.1% trypsin made up with SM containing 0.02% EDTA.

4. After a few seconds, stop the trypsinization by decanting the SM-EDTA medium and add CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium. Transfer the cell suspension to a 15-ml plastic conical centrifuge tube.

5. Centrifuge at 650 × g for 7 min; decant supernate.

6. Resuspend the pellet with CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium, and seed equally into four 25-cm² flasks, or one 75-cm² flask. We use 5 ml of medium containing the cell suspension. Cells should be seeded at high density.

7. After 10 min, gently swirl dishes or flasks to encourage cells to adhere to each other.

8. Return the cultures to the incubator and do not disturb for 3 to 4 days.

9. After 3 to 4 days, examine the cultures for growth of epithelial colonies.

DISCUSSION

There are several critical steps in the procedure that will mean the difference between success and failure. During the initial phase of collection of the tissue, make sure that the pathologist or surgeon does not place the samples into phosphate buffered saline (PBS) or physiological saline, such as Kreb's solution. The carrier medium defined here will keep the tissue viable up to 4 or 5 days at a 95% efficiency. Do not use penicillin-streptomycin, nystatin, mycostatin, amphotericin B, tetracyclines, etc.; they will kill the cells.

All plastic ware, i.e. petri dishes and 75-cm² flasks, should be kept in a constant environmental room at 72° C at a relative humidity (R.H.) of 75%. The flasks under these conditions will remain in acceptable condition for 2½ years. The single well and multiwell dishes can be used for up to 6 months when stored under these conditions. All pipettes used to pipette cells should have an aperture of 1.5-mm diameter. The FBS must be evaluated before use (1). Collagenase and purified trypsin prepared specifically for tissue culture applications should be screened for mycoplasma (2). Photographs of vertical stratification of epithelial patches and tables of growth kinetics are presented in Milo, Ackerman and Noyes (4).

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FELINE SARCOMA VIRUS INDUCED IN VITRO PROGRESSION
FROM PREMALIGNANT TO NEOPLASTIC TRANSFORMATION
OF HUMAN DIPLOID CELLS

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SUMMARY

Human diploid cells morphologically transformed by feline sarcoma virus were serially propagated under selective cell culture conditions. When injected into nude mice prior to passage in soft agar (0.35%), morphologically transformed cells did not produce tumors. However, when propagated under selective cell culture conditions, transformed cells grew in soft agar and, when injected subcutaneously into the subcapsular region of the nu/nu mice, produced neoplastic nodules histopathologically interpreted as fibromas. Karyological examination of cell populations grown out from the tumors confirmed that the tumors were composed of human cells. Examination of electron micrographs of the excised tumor tissue revealed the presence of budding virus particles. Tumor cells isolated from nude mice and morphologically transformed cells both contained the feline oncornavirus-associated cell membrane antigen. It was concluded that expression of feline oncornavirus-associated cell membrane antigen is associated with an early stage of feline retrovirus-induced carcinogenesis, namely focus formation. In addition, it was shown that FeLV-FeSV can induce morphological transformation in human cells in vitro and that there is a requirement for the cells to passage through soft agar before subsequent tumor formation (neoplastic transformation) can be demonstrated.

Key words: Feline sarcoma virus, neoplastic transformation, human diploid cells.

INTRODUCTION

Feline retroviruses have been reported to morphologically transform cells of numerous animal species including hamsters (1-4), cats (5-7), dogs (2,4,8,9), pigs (2,5), sheep (10), monkeys (11), and humans (4,7,9,10,12,13,14,15). However, rat and mouse cells are refractory (16) along with WI-38 cells (17) to feline retrovirus transformation and thus there are no reports about neoplastic transformation, i.e. ability to produce tumors with the in vitro transformed cells in an appropriate animal host.

Although the role of feline retrovirus in spontaneous neoplastic diseases of various heterologous animal hosts is not known, the feline sarcoma virus (FeLV/FeSV) induces fibrosarcomas in cats (5,6,18) and other species. The oncogenic properties of FeLV/FeSV in humans are not known.

The objectives of this study were to determine the optimal in vitro conditions of transformation of human diploid cells by FeLV/FeSV and the oncogenic potential of these transformed cells in nu/nu mice.

MATERIALS AND METHODS

Primary NFS Cultures. Primary human cell cultures (NFS) established from foreskin tissue as previously described (19), were maintained on Eagle's Minimal Essential Medium (MEM)-Hank's buffered salt solution (HBSS)-25 mM Hepes buffer at pH 7.2 (Gibco, Grand Island, N.U.), 1 mM sodium pyruvate, 2 mM glutamine, 50 μg gentamycin per ml (Schering Diagnostics, Port Reading, N.J.), 0.2% sodium bicarbonate, and 10% fetal bovine serum (FBS) (Biofluids, Inc., Rockville, MD).

Preparation of Stocks of Snyder-Theilen FeSV. Stocks of ST-FeSV were prepared as previously described (20). Feline embryo cells at 50-70% confluency were treated with DEAE-Dextran (40 μg/ml) in L15 medium (Gibco, Grand Island, N.Y.) at room temperature for 20 min. Following removal of the DEAE-Dextran, a virus inoculum in L15 medium supplemented with 5% FBS was added to the cell sheet for 2 hours. The cell sheet was then refed with L15 medium + 15% FBS and incubated for 7-10 days at 37°C in a 4% CO_2 enriched atmosphere. The cells were harvested by scraping and subjected to two cycles of rapid freezing in dry ice/95% ethanol and thawing in a 37°C water bath. The cell suspensions were centrifuged at 350 x g for 10 min; the supernatant solution was filtered through a 0.45 μm Millipore filter and stored in 0.5 ml aliquots at -70°C.

Selection of "Pure" Populations of FeSV-infected Human Cells. Preconfluent cell populations at population doubling (PDL) 4-14 (1) were seeded at 0.5-1.0 X 10^4 cells per sq cm in MEM + 10% FBS. After each split at a 1:4 split ratio, the PDL were increased by 2. After 24 hours, the cells were inoculated with 1.250 dilution of FeSV which had a titer of 6.1 X 10^4 ffu per ml. The protocol for inoculation was identical to that used on the human diploid cell cultures except that MEM growth medium was used in place of L15. Ten days later, the cultures, which contained 10 foci per sq cm for a

20 sq cm plate, were passaged 1:4. Those areas in the confluent culture containing hyperrefractile cells that stained densely with hematoxylin were identified as foci (12). The cultures attained confluency in five days and were serially passaged thereafter 1:10 until "pure" populations were attained that contain 100% Feline Oncornavirus Cell Membrane Antigen (FOCMA)-positive cell populations. These infected cell populations required 8-9 days to reach saturation density.

Effects of Different Growth Media on Proliferation of FeSV-infected Cells. Cells were seeded at 1:10 dilutions into different growth media supplemented with 10% FBS. Cell proliferation was then monitored in either McCoy's 5A, MEM-Mg^{+2}, MEM (Gibco, Grand Island, N.Y.) or EBM LoCal (Biolabs, Northbrook, Ill.). McCoy's 5A medium was used previously for the growth of feline leukemia virus transformed (FL-74) cells in suspension culture (20); MEM-Ca^{2+} - Mg^{2+} and EBM LoCal were selected because these elements have previously been found to alter Adeno-12-induced focus formation of hamster embryo cells (21), and to alter susceptibility and refractoriness of Yaba tumor pox virus-induced focus formation (22).

Release of Infectious Virus from FeSV-infected Human Cells. Pure populations of FeSV-infected human cells growing on either EBM LoCal, McCoy's 5A, or MEM media were assayed for the release of infectious virus. Twenty-four hours after seeding at PDL 20, or as the cells stopped proliferating, aliquots of the supernatants were removed and filtered through a 0.22 μm Swinnex Millipore filter, then diluted and used as inoculum. Ten days later, the infected plates were fixed in 10% formalin and stained with hematoxylin and eosin. Finally, the foci were enumerated. These foci contained cells that were morphologically distinct from the normal cells (see above) and are hereafter referred to as morphologically transformed cells.

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Passage of Morphologically Transformed Cells Through Soft Agar. Soft agar was used as a suspension medium for the growth of morphologically FeSV transformed cells. A 2% agar base, RPMI 1629 medium (Gibco, Grand Island, N.Y.) was supplemented with 20% FBS. Transformed cells were harvested from the supernatant and resuspended in EBM LoCal medium + 20% FBS, 1% essential amino acids, 1% essential vitamins, 0.35% agar, 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate and 50 µg/ml gentamycin. Two ml of this cell suspension were seeded at 1-2 X 10^5 cells/ml over the agar base plates. These media were used because the morphologically transformed cells grow more optimally in these media. We have tried PHMI 1640, MEM, BME, etc., with a lesser extent of success.

FOCMA Detection. An indirect immunofluorescence test for FOCMA (23) was performed on the morphologically transformed cells. Proliferating transformed cells from either monolayers or soft agar were harvested by centrifugation at 650 x g for 7 min at a cell density of 0.5-1.0 X 10^6. The reference primary reagent (cat serum) used in this study was from a FOCMA antibody positive cat that was persistently viremic. This agent was shown to be specific for FOCMA, since absorption of the serum with intact and ether-disrupted FeLV (10" purified particles per ml of serum) did not decrease antibody titers (24). In addition, this reagent produced membrane fluorescence on FeSV-infected human neonatal foreskin cells, but not on uninfected human foreskin cells (unpublished data).

Histopathology and Electron Microscopy. Tumors of FeSV-infected cells and FeSV-infected cell populations prepared from boluses growing in soft agar were prepared for histopathology and electron microscopy.

Athymic nude (nu/nu) mice, backcrossed 5 or 10 times which were obtained from Sprague-Dawley, Madison, Wisconsin were selected for evaluation of the neoplastic

potential of FeSV-transformed human cells. Preconfluent cultures of FeSV-transformed cells were prepared for injection by scraping with a rubber policeman and were pelleted by centrifugation at 540 x g for 7 min. The pellet was resuspended in fresh MEM and recentrifuged. After resuspension in MEM + 0.5% agar, 0.53-1.0 X 10^7 cells were injected subcutaneously into athymic nude (nμ/nμ) mice which had been irradiated 3-4 days previously with 450 rads ^{137}Cs γ-rays. The nodules which developed at the site of inoculation were excised after six weeks' growth, fixed in 3% glutaraldehyde-0.1 M cacodylate buffer at pH 7.4, then prepared for histopathology and electron microscopy. In addition, FeSV-infected proliferating populations isolated from boluses obtained from soft agar were scraped from the substratum of the flasks, pelletized by centrifugation at 650 x g for 7 min, and fixed in 3% glutaraldehyde - 0.1 M cacodylate buffer at pH 7.4 for examination under an electron microscope.

Karotype Analysis of Excised Tumors. Tumors from 0.8 - 1.2 cm in length were identified at the site of injection after 6 weeks. These tumors were surgically removed from the nude mice and cell suspensions were made as described elsewhere (19).. Hyperimmune antiserum prepared against nude mouse skin cells was added to the culture of 500,000 tumor cells in a 75 sq cm flask at 0.6 ml per 15 ml of growth medium. The medium containing the antiserum was renewed every 24 hours. Seventy-two hours later, the proliferating cells were refed with 5 μg/ml of colcemide (Gibco, Grand Island, N.Y.) in 10 ml of growth medium and incubated for 3 hours at 37°C. The medium was decanted after 3 hours and the cell monolayer was rinsed with warm PBS-Ca^{2+} - Mg^{2+}. Following their removal with 10 ml of trypsin-versene solution (40:1), the cells were recovered by centrifugation at 650 x g. The cell pellet was fixed in cold methanol-glacial acetic acid (3:1). A suspension was dropped onto a glass slide, dried and stained with prefiltered 5% Giemsa solution.

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RESULTS

<u>Selection of Pure Populations of FeSV-infected Cells.</u> At five to 10 PDL after starting selective subculturing of the virus-infected human cells was started, it became impossible to distinguish individual foci in the culture because of the increased number of infected cells in the population. Many hyperrefractile round FeSV-infected cells were released into the growth medium. Populations of $1-2 \times 10^4$ cells/ml were harvested from this "breeder" culture. Two methods of harvesting these free-floating cells resulted in the selection of two cell types. Centrifugation and resuspension in fresh MEM + 10% FBS gave rise to cells which attached to the substratum and exhibited a variety of cellular morphologies (Fig. 1). Direct transfer of the old media and cells into a flask produced a seeding suspension; some of these cells attached to the substratum, while others began to grow into large boluses in suspension which varied in size and contained from 25-200 cells (Fig. 2). Both harvesting methods gave rise to cultures containing pleomorphic cellular and colony morphology.

<u>Growth Characteristics in Different Culture Media.</u> We have tried many recipes for media. Table 1 lists their ability to support the growth of the transformed cells. MEM-Mg^{2+} + 10% FBS, McCoy's 5A + 10% FBS, and McCoy's 5A supplemented with 5 ug/ml spermidine + 10% FBS supported cell growth for less than 3 PDL. McCoy's 5A - 5 µg/ml uridine - 10% FBS supported growth for 3-5 PDL.

FeSV-infected cells subpassaged 1:10 into EBM LoCal medium plus 10% FBS proliferated for 6 PDL; however, further subpassaging resulted in cell lysis. Cultures that were subpassaged on MEM-Hepes died after 42-46 PDL.

<u>Release of Infectious Virus.</u> The supernatants were individually assayed for infectious virus (see Materials and Methods) after 20 PDL or when the cells ceased proliferating and lysed.

The data in Table 2 indicate that only MEM and EBM LoCal supported the production and release of infectious virus.' There were 350 times more ffu released from cells grown on MEM than from cultures grown on LoCal. This suggests that virus production and release, like cell proliferation, is dependent on the specific cell culture medium used. Calcium has also been shown to alter virus-induced cell morphology and to produce biochemical changes in other virus-cell systems (15, 25). Cell populations grown in McCoy's 5A supplemented growth medium died after a short time. There was no release of detectable infectious units.

Growth in Soft Agar. Single cell clones were obtained by cloning single cell suspensions in agar to evaluate the infected cells for their ability to grow in soft agar. Free-floating cells were seeded at $1-2 \times 10^5$ cells/ml into soft agar after 16-20 PDL and round compact colonies observed 10-14 days later containing 50-100 cells per bolus (Fig. 3). The frequency of bolus formation was 2.5 to 5×10^{-4}.

FOCMA Expression on FeSV-infected Cells. Randomly proliferating cells were assayed for the presence of FOCMA by indirect immunofluorescence (26). Figure 4 (A) is a light micrograph of several infected cells. Figure 4 (B) illustrates the fluorescent pattern of FOCMA on the same cells. The pattern is typically patchy. We observed a similar pattern of FOCMA fluorescence by FL-74 cells grown in spinner flasks (20). To date, all transformed cells treated with fluorescein isothiocyanate tag control cat serum have been negative.

Growth of FeSV-transformed Human Cells in Nude Mice. To evaluate the neo-plastic potential of these FeSV-transformed proliferating human cells, $0.5-1.0 \times 10^7$ cells were injected into nude mice. Twenty-four hours later, the bleb at the injection site regressed. After 5-30 days, palpable nodules were evident. They increased in size

to 0.8 - 1.4 cm over a four-week period at the end of which the nodules were excised and prepared for histopathology.

Histopathology of Tumors from Nude Mice. Histologically, the nodules from both mice were well encapsulated, sharply demarcated cellular masses (Fig. 5). The neoplastic cells were ovoid to spindle-shaped and contained a single vesiculated nucleus usually with a prominent nucleolus. The cytoplasmic margins were frequently indistinct and some cells appeared to form syncytia. An application of the Massons trichrome stain showed the eosinophilic fibrillar intercellular material to be collagen. A basophilic (hematoxylin) mucin-like intercellular material was admixed with the collagen fibers and stained with Alcian blue. This revealed the presence of acid mucopolysaccharides (Fig. 6). Mitotic figures were rare, the mass was well vascularized and the neoplastic cells were located immediately adjacent to the vessels. The mass was interpreted to be a fibroma.

Under electron microscopic examination, cells in culture and cells from the nodules removed from mice were both found to contain virus-like particles (Fig. 7).

Distribution of Chromosome Number in Tumor Material. Tumors excised from nude mice were grown in vitro as described previously. In no case of the metophase spreads from 5 different tumors evaluated was there significant deviation from the diploid number of chromosomes.

DISCUSSION

Replication of FeLV-FeSV in human cells and subsequent focus formation were demonstrated in this study, confirming previous reports (9,10,15,26,27,28,29). The data obtained in this study, however, suggest that focus formation (i.e. morphological transformation) (2) represents only a transitional stage in the neoplastic transformation process. It is interesting to note that Azocar and Essex (17) did not observe morphologically transformed cells when WI-38 were infected with FeSV. However, if we add fungizone or penicillin - streptomycin to the cultures much in the same manner they did, no foci were observed. This study showed that the separation of morphologically transformed from nontransformed normal cells and growth and passage in soft agar were prerequisites for the demonstration of neoplastic properties by the transformed cells. The separation was accomplished by culturing and passaging the cells in a low-calcium supplement growth medium for 2 PDL to 5 PDL in MEM-Hepes EBM-LoCal medium. The resultant transformed cell populations grew in suspension, while populations that contained normal-appearing cells did not. Selective culturing in suspension of these cells in EBM-LoCal medium followed by subculturing in MEM 5 PDL later resulted in the cells reattaching to the substratum. It was necessary to serially subpassage these cells for an additional 16-20 PDL in 1 X MEM-Hepes growth medium before they would grow in soft agar. The morphologically transformed cells, when passaged through soft agar, formed colonies of 50-100 cells per bolus at a frequency of 2.5 - 5 X 10^{-4} per 25 sq cm. These cells, isolated from soft agar and grown in a selective medium, were injected into 6-week-old nude mice to evaluate the oncogenic potential. Nutritional requirements and time in culture appear to determine growth and expression of the neoplastically transformed cells. Selective nutritional requirements have also been shown for other FeSV-transformed cell systems such as

transformed feline producer cells which grow optimally on McCoy's 5A (22) or Ad-12-transformed hamster cells which require a low-Ca^{+2} growth medium (21). The transformed human cells that also grew on EBM-LoCal medium did not grow on MEM minus Mg^{+2} - Ca^{+2}.

Tumors produced in the nude mice (0.8 - 1.4 cm in size) were found to contain collagen and were interpreted histopathologically to be fibromas, not fibrosarcomas (25). Removal of the tumor was followed by growth of the cells in culture; subsequent karyological examination indicated that the tumor cells were of human origin. Examination of electron micrographs of excised tumors and proliferating cells from in vitro populations of morphologically transformed cells, and examination of cells passaged through agar, revealed that both types of cells were shedding virus particles. Assay of the morphologically transformed cell populations (30) before and after growth in soft agar revealed that FOCMA was present at both stages in the transformation sequence. These results suggest that FOCMA expression is associated with the early events in the transformation process. There appears to be a program or staging process that must occur in FeVL/FeSV-transformed human cells before they will produce tumors when injected into a suitable host. The results reported here appear to be similar to the multistage process of chemical carcinogen-induced transformation (25). FOCMA and virus expression appear to be associated with early stages of morphological transformation, while growth in soft agar is associated with a later transitional stage. A high correlation exists between growth in soft agar and tumor formation. It was noted that while virus-infected cells would not produce tumors prior to their growth in soft agar, after they were passaged through the soft agar they would produce tumors. Again, this suggests that growth in agar acted as a selection process to permit the proliferation of cells that can produce tumors when inoculated into a

suitable host. Therefore, we have shown that FeSV neoplastically transformed cells are capable of producing tumors in nude mice.

The release of the infectious virus from human cells that form tumors or grow in soft agar raises the question of possible horizontal transmission. FeLV and FeLV/FeSV have been shown to be horizontally transmissible among cats (30) and FeLV, under laboratory conditions, was horizontally transmissible to dogs (15). A serological survey indicated that no antibody to FeLV occurred in hundreds of individuals exposed to viremic cats (12,31). However, Jacquemin et al. (32) reported that purified human IgG from patients with chronic myelogenous leukemia specifically neutralized reverse transcriptase isolated from FeLV.

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ACKNOWLEDGEMENTS

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Growth of FeSV-Transformed Cells in Different Growth Media

Table 1

	Growth Medium	PDL
(1)	MEM + 25 mM Hepes	46
(2)	MEM - $Mg^{+2}Ca^{+2}$	<3
(3)	McCoy's 5A	0
(4)	McCoy's 5A + 5 µg/ml spermidine	<2
(5)	McCoy's 5A + 5 µg/ml uridine	4
(6)	EBM LoCal	7

Table 1

The proliferative characteristics of a pure FeSV-transformed human cell population were measured as PDL on different types of growth media. Other experiments on other populations for PDL 1 through 6, not reported here, supported these data. In most cases the mean PDL did not exceed one sigma S.D. for values reported here. PDL as referenced in the text is: 1 serial subpassage at 1:2 split ratio at 95% absolute plating efficiency. One population doubling (PDL) is that cell population that was serially passaged 1:4 at confluency. If the cells were serially passaged at 1:4 they had proceeded through PDL.

Measurement of Focus Forming Units on Appropriate Monolayer Cultures.

Table 2

	Growth Medium	$ffu/75\ cm^2\ flask^*$
(1)	MEM + 25 mM Hepes	$1.9 \times 10^7 \pm 1.4 \times 10^5$
(2)	MEM-MG^{+2}-Ca^{+2}[**]	——
(3)	McCoy's 5A[**]	——
(4)	McCoy's 5A + 5 µg/ml spermidine[**]	——
(5)	McCoy's 5A + 5 µg/ml uridine[**]	——
(6)	EBM LoCal	$5.5 \times 10^3 \pm 1.1 \times 10^3$

[*] Dashes (——) mean that ffu were not detected when supernatant solutions were assayed on appropriate human monolayer cultures (19) (Morphological Transformation) after the cell populations ceased to proliferate and lysed (Table 1).

Table 2

These figures show the results of a focus-forming assay of FeSV-transformed cells cultured in different growth media. NFS cells were originally infected with a 1:250 dilution of FeSV from a pool with a titer of 6.1×10^4 ffu/ml. The virus assay period was determined to be at least 20 PDL at 1:10 split ratios after virus infection for cells grown on MEM-Hepes medium. This time period was selected in order to ensure that only pure populations of virus-transformed cells were assayed. Focus-forming units/75 sq cm flask were determined for $1.5-2.0 \times 10^6$ cells per experiment. These data represent the results for n of 4 for MEM at PDL-20 and n of 3 for LoCal at PDL 7. They are presented here as mean values $\pm$ 1 sigma standard deviation.

** Cells cultured in these media exhibited limited proliferative capability and ceased proliferating prior to the virus assay period.

Modality of Human Chromosomes of Cells Prepared from Tumor Cells.

Table 3

PDL [*]	Model No.	Range
10	45	43-47
10	43	42-45
5	46	45-47
.7	46	45-48
6	45	44-46

* These PDL represent proliferation of population of FOCMA positive cells seeded from the tumor. The range represents the distribution of chromosomes seen on a single slide.

Table 3

The data presented here represents our attempt to count the numbers of human chromosomes found in the cells that produced tumors in the nude mice. The tumors were removed seeded _in vitro_ and at subsequent PDL evaluated.

Figure 1

This photograph shows proliferating FeSV-transformed human cells. A super-natant cell suspension from a culture at PDL 17 containing 70-90% transformed cells was harvested by centrifugation at 650 x g for 7 min and the cell pellet was resuspended and seeded in MEM + 10% FBS. Other nonattached cells remain rounded up and floating in the medium. Magnification X40.

Figure 2

A cell suspension recovered without centrifugation from the overlying growth medium of FeSV-transformed human diploid cells, PDL 16, was seeded directly into a flask without replenishing the growth media. Ten days later actively dividing boluse: of cells were observed. Magnification X32.

Figure 3

A suspension of free-floating viable cells in the overlying growth medium from a FeSV-transformed culture at PDL-12 was harvested by centrifugation at 650 x g for 7 min. Cell populations at $1\text{-}2 \times 10^5$ cells/ml were seeded into 0.5% soft agar containing LoCal growth medium. Ten days after seeding, colonies of cells were observed. Magnification X82.

Figure 4

Proliferating FeSV-transformed cells, $0.5-1.0 \times 10^6$, were harvested by centrifugation at 650 x g and incubated with FOCMA antibody reference cat serum, obtained from an animal with a regressing sarcoma, for 30 min. Cells were washed free of unbound cat globulins with three consecutive rinses of Hank's balanced salt solution and incubated with a 1:20 dilution of goat anti-cat gamma-globulin (Sylvania, Inc., Milburn, N.J.) which had been conjugated to fluorescein isothiocyanata. (A) Light micrograph of FeSV-transformed cells, (B) UV-fluorescence micrograph of the same field. Magnification X250.

Figure 5

A well-encapsulated subcutaneous nodule in an nµ/nµ mouse. The cells are individually aligned (arrow) or arranged in syncytial sheets (s). Magnification X10.

Figure 6

Electron micrograph of cell from subcutaneous nodule in nµ/nµ mouse. The interstitium contains flocculent electron-dense material (arrowhead) and numerous fibrils (arrow) containing the characteristic 640 $\overset{\circ}{A}$ banding of collagen. Magnification X45,000.

Figure 7

Virus particles (arrows) budding from cell membrane of fibroblast in culture prior
to injection into nμ/nμ mouse. Magnification X28,000.

Ultraviolet Radiation Induced Neoplastic Transformation

of Normal Human Cells, In Vitro

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Abbreviations: UV, ultraviolet 254 nm radiation; E.D. 50, effective dosage 50%; PDL, population doubling; CM, complete growth medium; FBS, fetal bovine serum; PBS, Dulbecco's Phosphate buffered saline at pH 6.8; $J \cdot m^{-2} \cdot sec^{-1}$, Joules per meter^2 per second^1; S phase, scheduled DNA synthesis; RCE, relative colony forming efficiency.

SUMMARY

Human foreskin cell cultures in S phase of the cell cycle were exposed to UV radiation at a dose cytotoxic to 50% or less of the cells, in the presence of insulin. Cell populations treated at a dose less than E.D. 50, when selectively subpassaged in a high amino acid supplemented CM after 20 PDL, were able to grow in soft agar. Cell populations 5 PDL after treatment exhibited altered lectin agglutination patterns but would not grow in soft agar. UV-treated cell populations also grew in a reduced serum concentration and at 41°C. These indices, along with abnormal colony morphology, appeared to be associated with early events in the expression phase of the transformed phenotype. After 20 PDL in the selective CM we observed a frequency of 20 colonies in 10^5 cells seeded in soft agar. The cell populations derived from these colonies, when propagated and injected into the nude mice, formed myxofibromas at the injection sites rather than the type of tumor (fibrosarcoma) previously described for chemical carcinogen-induced neoplasms.

INTRODUCTION

Carcinogen-induced transformation of human cells _in vitro_ by chemicals or irradiation has been difficult. Refractoriness of human cells to _in vitro_ transformation is not unique. Recently, however, neoplastic transformation of human cells by chemicals (1) and viruses (2) has been achieved. In addition, Sutherland (3) using U.V. treatment of human cells damaged the DNA and transformed the cell populations to an anchorage independent state of growth. It was necessary to use multiple treatments at subtoxic doses of U.V. to damage the DNA and induce transformation. Other studies with refractory human cells have indicated that carcinogens can induce unscheduled DNA synthesis (4) or repair synthesis (5). However, these reports do not attempt to correlate damage to DNA with expression of carcinogenesis. Instead, Heflich (4) correlated removal of chemical carcinogen and induced damage to DNA with cytotoxicity (4). In this report, we have expanded on our preliminary report of U.V. induced neplastic transformation of human cells (7) and present data here on the reproducibility of the process. We also present data on the interrelationship between anchorage-independent growth and tumor growth as the normal cells pass through a retrodifferentiated sequence from induction to neoplasia.

MATERIALS AND METHODS

We have found that in order to observe reproducible transformation of human cells exposed to U.V. it was necessary to first use low passage cell populations (PDL 1-5) and, second, to complete a cell survival curve prior to the selection of an appropriate treatment dose.

Cell Cultures

Primary cell cultures were obtained from neonatal foreskins (NFS) as previously described (8) and maintained on CM, viz., minimum essential medium (MEM; 25 mM HEPES, GIBCO, Grand Island, New York) at pH 7.2 supplemented with 10% FBS, (Sterilin, Kankakee, Illinois); 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate and 50 ug/ml Gentomycin, in an atmosphere of 4.0% CO_2-enriched air at 37°C.

Survival Studies of Irradiated Cells.

Immediately after UV treatment and/or 40 h later, cell survival was measured by either dye exclusion (9), colony forming ability (10), or incorporation of ^{3}H-thymidine into cellular DNA (9). After seeding, cultures were irradiated with a 15W Germicidal Electric lamp (15GT8) at a fluence rate of 1.2 $J \cdot m^{-2} \cdot sec^{-1}$. The fluence rate was measured by a Blak-Ray UV meter (UV Products, International, San Gabriel, Calif.). If we wished to measure colony forming capability of the treated cell population, one thousand cells were seeded in 25 cm^2 wells (Falcon Plastics, Oxnard, Calif.) and fed with CM supplemented with 20% FBS. These cultures were incubated at 37°C in a 4% CO_2 enriched air atmosphere for 9 days, fixed in phosphate -3% formalin stained with Hematoxylin-Eosin, and enumerated under 23X. If the effect of the treatment on cell survival was to be measured by dye exclusion, approximately 5000 cells $-cm^{-2}$ in a 25 cm dish were trypsinized at the conclusion of the UV treatment and counted in a cytometer (9). Inhibition of the incorporation of (^{3}H)-CH_3-thymidine (S.A. 54.0

Ci/mmole) into the DNA of UV treated cells was measured by sampling the cell population from 0-48 hrs following initiation of UV treatment (8). At the sample times 1 hr intervals) three coverslip cultures were removed, fixed in 3:1 methanol:acetic acid solution, acid washed in 1.0N HCl and air dried. Two coverslips were transferred to scintillation vials and incubated overnight with 0.5 ml of NCS tissue solubilizer, neutralized with 1N NaOH. Ten ml of Insta-Gel (Beecham Inst., Palo Alto, Calif.) was added to each vial and the radioactivity counted in a Beckman scintillation counter.

Transformation

Once the cell survival studies were completed, we observed that all cell populations, when treated at a survival dose of 50%, optimize the formation of transformants.

The scheme of induction and selection of the transformed cells is presented along with the chronology of the events as they occurred (Fig. 1).

To maximize the number of UV-induced lesions going through scheduled DNA synthesis, the cultures were irradiated at the beginning of S phase (1). Preconfluent, logarithmically growing cultures at low (3-8) PDL were synchronized by placing them at a density of 5,000 cells-cm^{-2} into Dulbecco's Modified Eagle's Medium (Biolabs, Northbrook Illinois) at pH 7.2, supplemented with 10% dialyzed FBS but lacking arginine and glutamine. After 24 h, the amino acid deficient medium was replaced with CM to which 0.5 U/ml of insulin had been added. Ten h later, when the cell population was entering S phase of the cell cycle (1), the growth medium was removed, and the cultures were washed twice with Dulbecco's phosphate buffered saline, irradiated with 5.0 J m^{-2} UV and refed with CM containing 0.5 U/ml insulin. Upon completion of the treatment the cells were allowed to proceed through S phase, which required 8.2 h, and e mitosis. They were then split 1:2, then 1:10, into MEM supplemented with 10% FBS plus 8X nonessential amino acids (8X growth medium). The 8X growth medium inhibited

growth of cells that exhibited a normal phenotype (5), while allowing proliferation of transformed cells.

The transformed cell population at this time was identified as entering the early stage of the carcinogenesis process. During this stage of expression it is imperative that no part of culture remain in a saturation density growth state for more than 16 hrs prior to subpassaging the culture.

EARLY

Growth of cells at $41^{\circ}C$ and in medium with reduced serum concentration.

Cell populations, following treatment with UV, were seeded (5000 cm^{-2}) in CM and serially subpassaged at 1:4 on a 4-day schedule and maintained at $41^{\circ}C$ in a 4% CO_2-enriched air environment. Cell populations from companion cultures were seeded at 5000 cells-cm^{-2} in CM supplemented with 1% FBS, also in a 4% CO_2-enriched air environment.

Lectin agglutination

Treated and control populations at a 70-90% confluent density of proliferating cells were removed from the substratum of the flask by the action of 0.05% trypsin (8). Following centrifugation at 650 x g, the pellet was resuspended in PBS at pH 6.8 and recentrifuged twice. The final cell suspension was left on wet ice at a cell density of 10^6 cells -ml^{-1}. Wheat germ agglutinin prepared in PBS was added to the wells of microtiter plates (0.025 ml). To this, 0.025 ml of a suspension of 180,000 cells in PBS was added to each well and incubated at $21^{\circ}C$ for 10 minutes. The wells were then examined immediately.

TRANSITIONAL

Soft Agar

After serial passage of the UV treated cells for 20 PDL in 8X amino acid supplemented CM, 50,000 cells were seeded in 2 ml of 0.35% agar supplemented with

...odified Dulbecco's Lo-Cal medium (Biolabs, Northbrook, Ill.), overlaid on a 2 ml 2% agar base supplemented with RPMI 1629 growth medium enriched with 20% FBS. Colonies formed after 14-17 days' incubation in a 4% CO_2-enriched air environment at 17°C. These cultures were refed every 7 days with 0.5 ml of Lo-Cal medium-supplemental agar. Since a close correlation has been shown between the growth of chemically transformed human cells in soft agar and their neoplastic potential (1), growth of the UV-treated cells in 6-week-old male athymic nu/nu mice from a BALB/c background (Sprague-Dawley, Madison, Wisconsin) was chosen as a suitable assay system to determine the neoplastic potential of the cultures derived from UV-treated cells.

LATE'

Growth in nude mouse

Cells were harvested by trypsinization and resuspended in Dulbecco's EBM LoCal (Biolabs, Northbrook, Ill.) supplemented with 20% FBS, 1% essential amino acids and 0.35% agar. Six-week-old nude mice, which had been irradiated 3-4 days previously with 450 rad ^{137}Cs -rays, were injected subcutaneously with 0.5 X 10^7 UV-treated cells. Nodules became evident at the site of inoculation 14-21 days following injection and continued increasing in size. The blebs formed at the injection site regressed in 48-72 hrs.

RESULTS

Toxic response

In the early stages of our experiments, following the treatment with UV, we measured the toxic effect the treatment had on the UV-insulin treated cells by counting the total number of viable cells at 12 h (Fig. 2) immediately after initiating the UV treatment, or at 40 h after initiating treatment (Fig. 3). In addition, recovery of cell division from the toxic effects of irradiation, monitored by ^{3}H-thymidine incorporation into DNA and by cell counts, began at 44-48 h following the completion of the carcinogen treatment at an E.D.50 cytotoxic dose as measured by colony forming capability. The irradiated cultures not exposed to insulin exhibited a typical shoulder on the survival curve followed by a logarithmic decline at doses greater than 10 J. m^{-2}. However, cell cultures treated with 0.5 U/ml insulin and then irradiated lacked a shoulder at 10 J. m^{-2}, and were biphasic in nature. The toxic effect of increasing UV fluence was more sensitively measured by relative cloning efficiency (RCE) than by the trypan blue dye exclusion technique; it took roughly a 10-fold higher fluence to demonstrate a measured effect (Fig. 2) by dye exclusion than by inhibition of colony formation (Fig. 3). Nevertheless, whichever method we used to measure the response, the survival slopes were similar (Figs. 2,3). The response profiles of insulin-free cultures were similar to those reported by Lehman et al. (11) or Maher et al. (12) for skin fibroblasts from different normal individuals. We noticed that when insulin-treated cells were irradiated at 5 J. m^{-2} (Fig. 3) in S phase there was an increased survival rate of up to 120% over the insulin-free cultures.

Insulin-free non-UV treated synchronized cell populations exhibited an 18% RCE. Randomly proliferating cell populations treated with insulin-UV or UV alone, or synchronized cells treated with UV alone, exhibited a shoulder at fluences of 10 J. m^{-2} (Fig. 3). Insulin treatment appeared to increase sensitization of synchronized cell

populations to UV treatment at fluences of 10 J.m^{-2} up to 20 J.m^{-2}. The insulin–UV-treated cultures at 20 J.m^{-2} exhibited a RCE of 3%, compared to 6% for cells not exposed to insulin. However, the cultures which were exposed to insulin during the 40 h after irradiation did not exhibit a shoulder and the shape of the survival curve was multiphasic.

Growth characteristics of UV treated cells (Selective Process)

After 10–15 PDL in the 8X growth medium, the irradiated cultures appeared pleomorphic and contained more than 95% small polygonal cells, many of which were multinucleate and displayed multiple processes as seen by Contrast Interference Nomarski microscopy. Cells passaged from these cultures proliferated to saturation densities of 4–6 X 10^6 cells/75 cm^2 flask in 8X growth medium, whereas untreated NFS cells only reached 1.2–1.5 X 10^6 cells/75 cm^2 flask. Treated cells exhibited a loss of contact inhibition, a tendency to pile up in culture, and a pattern of irregular criss–cross growth. None of these altered characteristics were observed in control cultures. These cell populations were capable of subpassage to 120 PDL while the controls phased out after passage to 40 ± 5 PDL.

To examine the cultures for growth at elevated temperatures, populations were subpassaged 1:4 and incubated at 37°C in a humidified 4% CO_2 atmosphere to allow cell attachment to the substratum. The temperature was then raised to 41°C. As early as 10 PDL after irradiation, cell cultures derived from UV-treated populations actively proliferated at 41°C for 72 h, at which time they were 60–70% confluent. Control cells detached from the surface in less than 24 hrs.

Growth in reduced serum

Many transformed mammalian cells have been reported to grow at reduced serum concentrations (13,14,15). We examined our control and UV-treated human cell populations for this characteristic after 10 PDL by subpassaging into MEM

supplemented with 1% FBS. Table 1 represents the growth characteristics of these cultures at reduced serum levels. Control NFS cells ceased proliferating after 5 PDL. After an initially slow growth rate, the UV-treated cultures appeared to adjust to the new serum concentration, as shown by the reduction in time needed to attain confluency; they continued to replicate at least through 17 PDL in 1% FBS, at which time they were transferred back to MEM + 10% FBS.

Agglutination properties of UV-treated cells

After at least 10 PDL in 8X growth medium, control and UV-treated cells were tested for agglutinability by exposure to varying concentrations of wheat germ lectin. V-treated cells were agglutinated by 78 µg/ml of lectin, whereas control cultures of cells required 2500 µg/ml, more than 30 times as much (16).

Growth in soft agar

U.V. treated cell populations required 20-25 PDL in the selection medium prior to seeding in soft agar. This was in contrast to 16-20 PDL for chemical carcinogen treated cells (1). When U.V.-treated cells were serially passaged through 0.35% soft agar colony frequency was 20 colonies per 10^5 seeded cells. None of the untreated cells would grow in soft agar. Occasionally, we observed small colonies of 2-6 cells in size, but they could not produce colonies 50 cells or larger and were not viable when isolated from soft agar after a 14-day incubation period in soft agar (Table 2).

Tumor formation

The nodules which developed at the injection site in nude mice grew to be 0.6-1.05 cm in diameter and were well-encapsulated by mouse fibroblasts (Figure 4). Removal of the tumors followed by karyological examination (1) confirmed that they were of human origin. The centers of the nodules were necrotic with dense infiltration of neutrophils and occasionally contained areas with extensive cholesterol cleft formation. Blood

vessels were present within the nodules. The nodules were compatible with a benign

proliferation of exogenous cells and could most closely be described as myxofibroma-

like.

Of 6 mice inoculated with the same inoculum, 4 developed tumors. After 4-6

weeks of nodule growth, the animals were sacrificed by cervical dislocation and the

nodules removed for histopathology. These experiments were repeated twice. They

were fixed in 10% formalin, embedded, sectioned and stained with hematoxylin and

eosin. To date, similar results have been obtained in four successive attempts while 200

irradiated control animals have not formed tumors. To date, no tumors have been

observed when normal untreated cells were injected into the nude mouse (0 out of 10

inoculated mice).

DISCUSSION

Several parameters of putative changes in human foreskin cell populations during passage from the early stages of the transformation process to the late stages (growth in nude mice) were evaluated. Altered colony morphology (1), altered saturation density (1) growth at $41^{\circ}C$ and growth in 1% FBS-supplemented growth medium were all characteristics associated with UV-irradiated cell populations as early as PDL 5 after discontinuation of the carcinogen treatment. Altered colony morphology was observed within 3 PDL after discontinued treatment. Immediately after the completion of $\underline{S}$ (8.2 in length) the cells were split 1:10, and part of the treated cell population was cloned at 1000 cells $-cm^{-2}$. We observed colonies in the flasks or wells that exhibited a loss of contact inhibition and a criss-cross, disoriented piling up of the cells. These populations, when isolated from the rest of the colonies in the culture, gave rise to cell populations that exhibited abnormal morphology and an absence of the long parallel swirling growth patterns of normal untreated cultures.

After 10 PDL, irradiated cell populations exhibited an alteration in lectin agglutination profiles and grew at a temperature of $41^{\circ}C$. In fact, we found that 2-3 serial subpassages could be manipulated at $41^{\circ}C$. Normal cells will not survive 24 h at that temperature. However, at this point, the cells still would not grow in soft agar, nor were they able to produce tumors in nude mice. Serial passage to 20 PDL was required to produce populations that would grow in soft agar.

After passage through soft agar and tumor formation in the nude mouse, UV-treated cell populations were able to grow to a finite PDL of 120-140. Cells with a normal phenotype phased out at PDL 40 ± 5. It is interesting to note that UV light has been shown to induce carcinogenesis in the skin of man although it has weak penetrating ability. UV light can be differentiated from X-ray by the specific nature of the induced

damage and DNA repair processes. Owing to its particular features UV light, unlike many chemical carcinogens, does not require antecedent metabolic activation or specific binding before an interaction with cellular DNA.

The slopes of the toxicity profiles (survival curves) are similar to those seen for 10T1/2 cells (17) on a per cell basis (Fig. 2). When we evaluated the survival response patterns of cells at risk 12 and/or 40 h after initiating treatment (4–8 hrs prior to the reinitiation of scheduled DNA synthesis), by growing the cells at a low cell density, we found that the toxicity slopes (Fig. 3) were very similar to the slopes measured on a per cell basis (Fig. 2). We did note the presence of abnormal colonies and it was from these colonies that the neoplastically transformed cell populations arose. If we plotted the numbers of these abnormal colonies as a function of the dose in J m^{-2} recorded as a frequency relative to the number of normal colony phenotypes, the optimum numbers of transformants occurred at a survival dose -50 (E.D.50). At an E.D.65 the frequency of abnormal colonies was zero. The frequency of abnormal colonies we observed at E.D.25 was $10^{-3.5}$; at an E.D.50 it was $10^{-3.0}$; and at an E.D.60 it was $<10^{-5}$. These values were obtained for a single experiment. Repeat experiments give the same order of response. The same observations have been made for cell populations treated with chemical carcinogens of the type whose action does not require activation (1). As in animal cell systems, serially passaging these treated human cells 24 h after exposure of the cells to UV, to give them time to replicate, enhances the formation of abnormal foci.

Therefore, in order to produce cell populations in the initial stages of a transformed phenotype the transformed cells must be allowed to replicate. If the treated cells were permitted to remain in a confluent density or non-proliferating phase of growth after treatment, no transformed cell populations were obtained. Contrary to the observation by Little (18) that survival is enhanced when mouse cells are left in

g, growth-inhibited conditions after X-irradiation, human cells at that point are lost from f transformed phenotypic populations. However, the rate of repair processes in rodent cells is much slower than in human cells, which may explain why rodent cells may be allowed a longer repair time before they replicate and establish a transformed phenotype.

Since we can optimize the transformation events by irradiating NFS cells in S, while treatment in G_1, G_2 or M minimizes the events (Milo and DiPaolo, unpublished data), we believe that the process of UV-induced carcinogenesis in human fibroblast cells is complex, error prone, and subject to critical timing of exposure to UV at an appropriate dose of the physical carcinogen. Following a selection process, the expression stage can be controlled by allowing at least 20 additional rounds of proliferation to occur before the cells will passage through soft agar and form tumors in the mouse.

In conclusion, we agree that the rate of recovery of cloning ability of human fibroblasts after UV treatment shows a correspondence with the cells' ability to recover from the potential lethal effects of the irradiation (6). However, we have found that even though we can demonstrate UV-induced transformation at doses of E.D.50 or less, we cannot demonstrate transformation at higher doses. Therefore, we feel that cell populations contain subsets of cells that are susceptible to carcinogenesis but are lost from the total cell population by modulation of the normal phenotype upon prolonged exposure to the tissue culture environment. In addition, upon repeat of these experiments, at least 5 times we have found that: 1) none of the untreated cell populations exhibited a change in phenotype, 2) out of 5 different treated populations from 5 different tissue samples, 4 of the populations could be transformed by UV irradiation.

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               IN VITRO CHEMICAL CARCINOGENESIS AND CO-CARCINO-GENESIS IN HUMA--ETC(U)
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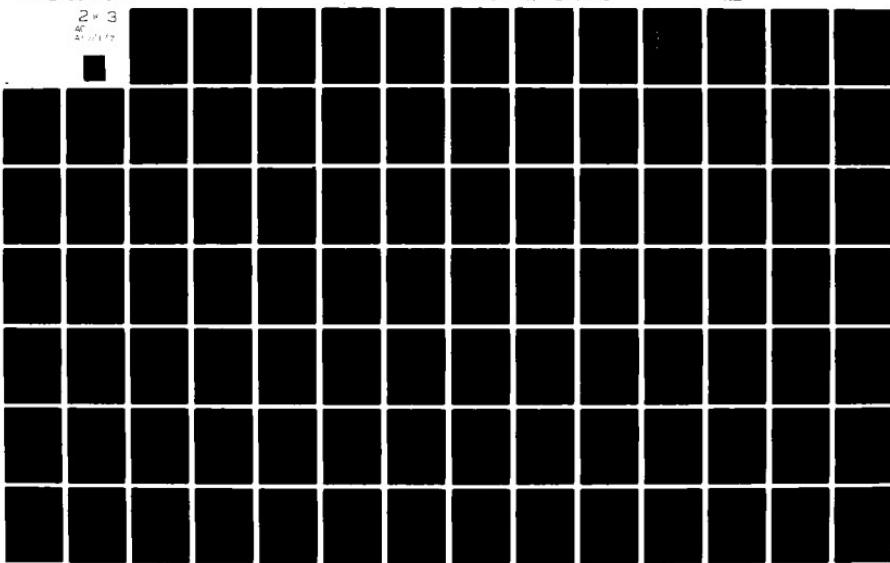
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Time (hr)	INDUCTION
	↓
0-24	Synchronization (PDL-5)
24-34	Sensitization
42.3	UV-Treatment
	↓
Week	SELECTION
	↓
1	Early
	PDL 6-10

Lectin Agglutination ← PDL 6-10 → Growth at 41° C / Growth in reduced serum

5	Transitional
	PDL 20-25
	↓
8	Growth in soft agar
	↓
10	Late
	PDL 34-40
	↓
14	Growth in nude mice (Tumor)

19
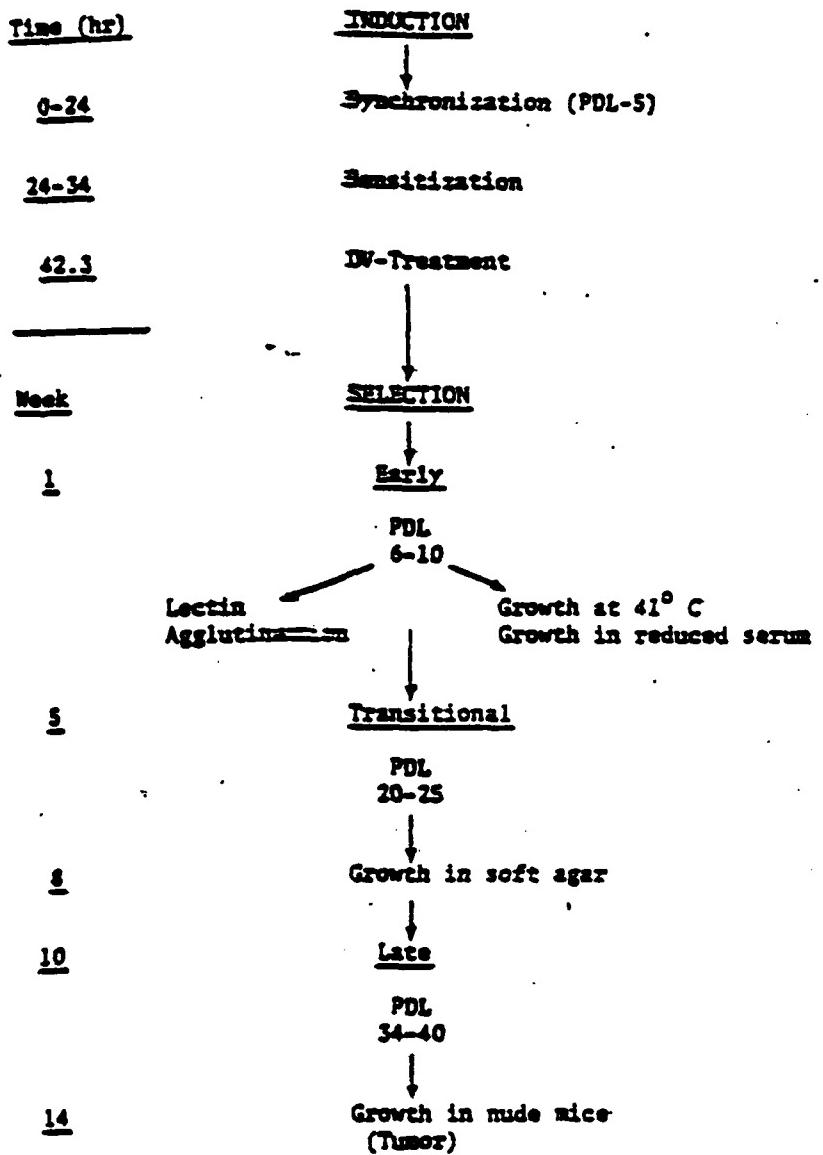
Legend Figure 1.

The scheme followed here is a graphic representation of the sequence originating
with the induction (treatment in early S) through tumor formation. Each critical stage
is represented by the PDL (population doubling) reached at each stage of the process.
Transition from colony formation in soft agar prior to growth in the nude mouse was
achieved following isolation and seeding of colonies in 75 cm^2 flasks to produce 5×10^6
cells of inoculum for each mouse.
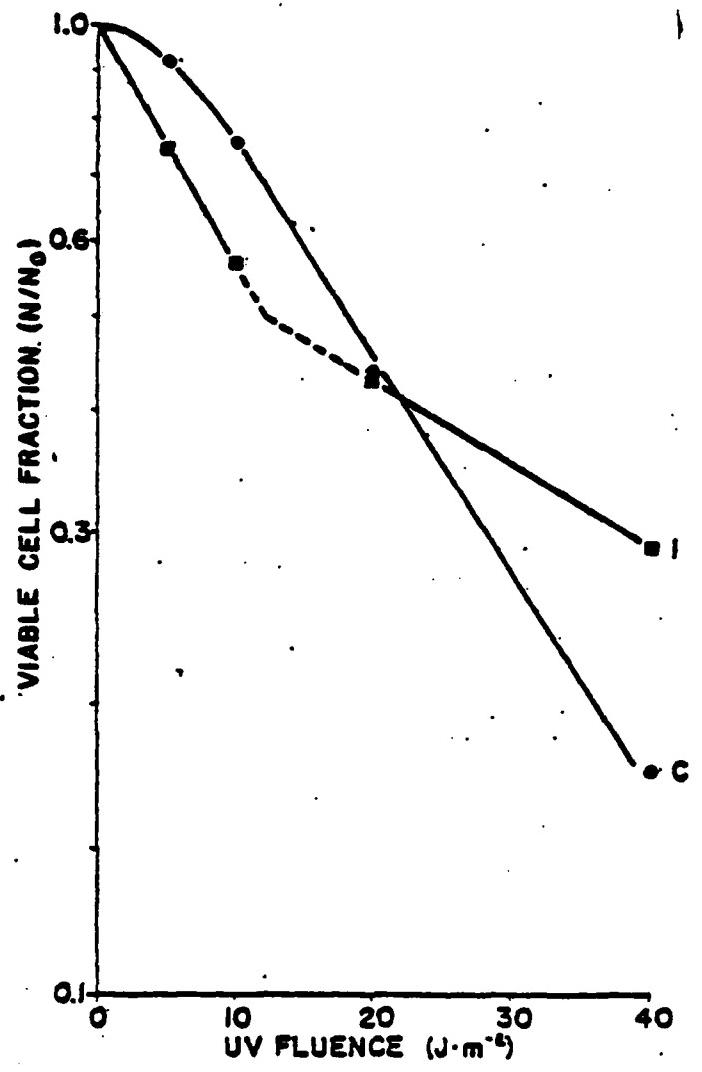
and Figure 2

Low PDL of NFS cells were seeded at 5,000 cells-cm^{-2} into 60 mm diameter, 4-1 plastic petri dishes and synchronized by amino acid deprivation. Following UV irradiation at a fluence rate of 1.2 J. m^{-2}-sec^{-1}, the plates were incubated at 37°C in a $\cdot$ CO$_2$ humidified air atmosphere. At this time cell suspensions were prepared by trypsinization and counted by trypan blue dye exclusion in a hemocytometer. The closed circles represent the irradiated cultures not exposed to insulin and the closed squares represent the irradiated cultures treated with 0.5 U/ml of insulin. Data points are the mean values for an n of 8.
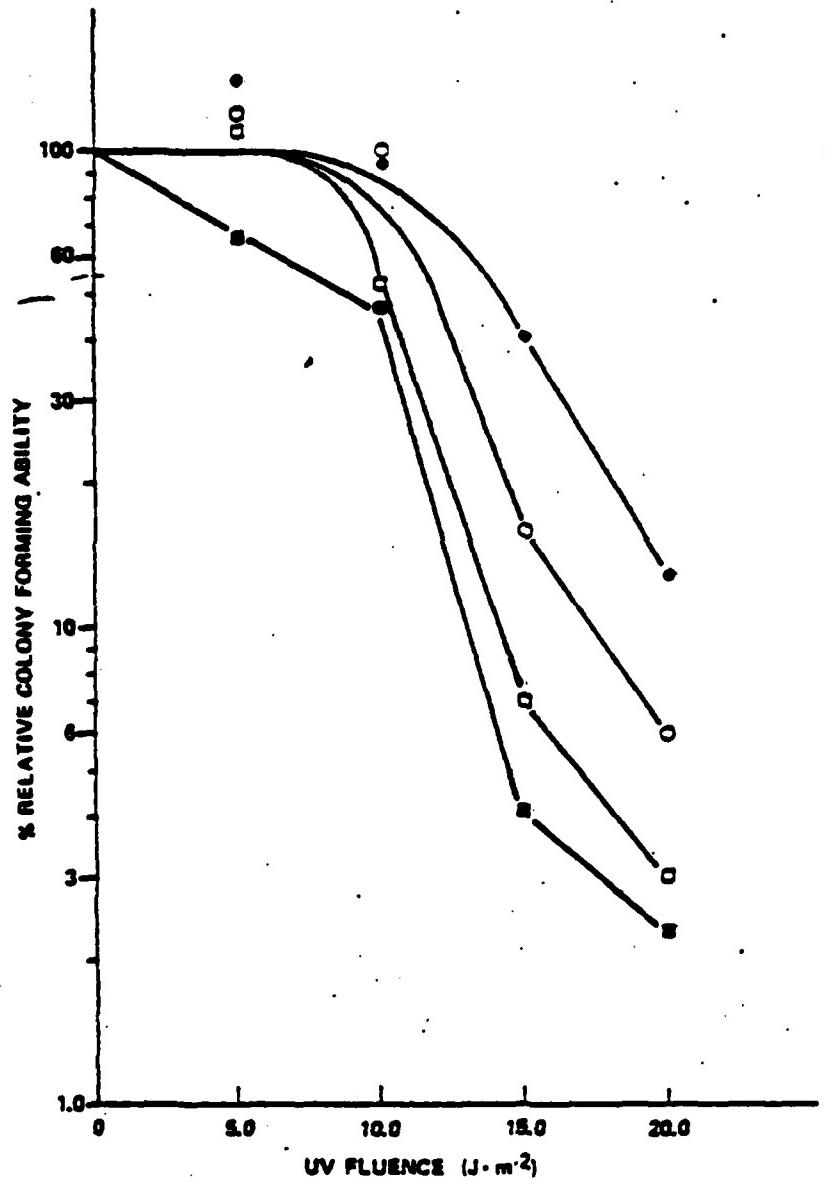
Legend Figure 3

Cells were seeded at 5,000-cm^{-2} into a 60 mm diameter 4-well plastic petri dish containing Dulbecco's Modified Eagle's Medium supplemented with dialyzed FBS, minus arginine and glutamine, at pH 7.2. These cells were released from the block (see text) and treated during the S phase with UV. Once the treated cells had completed S (8.2 h) they were seeded at a concentration of 200 cells-25 cm^{-2} well and cloned (3) for 11 days. The control (untreated) cultures exhibited 18-24% cloning efficiencies; results are reported as a percent of control values. The circles (o - •) represent cells treated with UV. The squares (□ - ■) represent cells treated with UV and 0.5 U of insulin. The open circles and squares represent time points 12 h post-treatment at the conclusion of the S+G$_2$+M part of the cell cycle, while the closed circles and squares represent time points coincident with the time points used for the trypan blue experiments (Fig. 1). Each point represents data from 10 different wells.
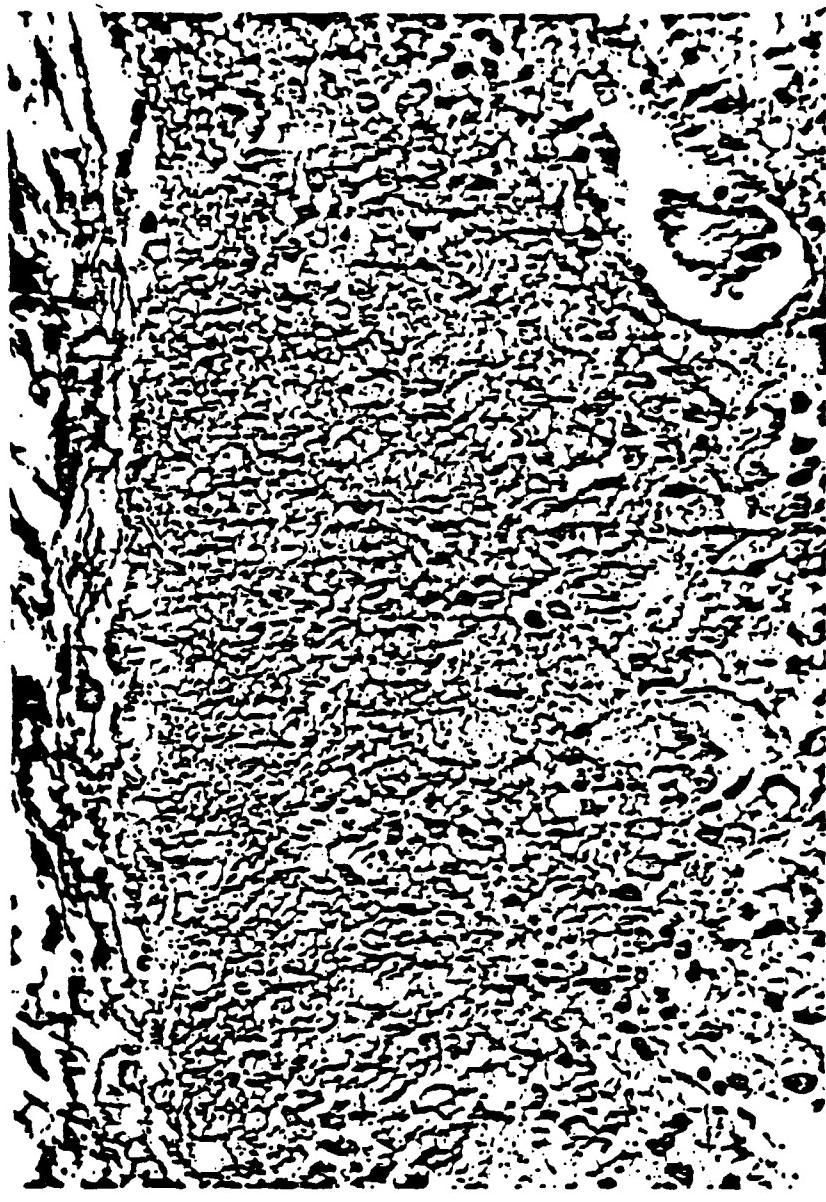
Legend Figure 4

Figure 4.

Photomicrograph (120X) of a serial section of a tumor excised from the subscapular region of a nude mouse. The tumor was excised 4 weeks after injection of 5 X 10^6 cells. The tumor was fixed in buffered formalin and stained with hematoxylin and eosin.

Legend Table 1

NFS cells (PDL 14) and UV-treated (PDL 17) passages were transferred into MEM
supplemented with 1% FBS and cellular proliferation was monitored. The NFS cultures
ceased dividing after 10 PDL; however, the cells from UV-treated culture appeared to
adapt to the low serum concentration.

Table 1. Growth of Control (NFS) and UV-Transformed
Human Cells at Reduced Serum Concentration.

SPLIT RATIO		DAYS TO CONFLUENCY		PDL	
NFS	UV	NFS	UV	NFS	UV
1:10	1:10	8	8	3.3	3.3
1:10	1:10	32	15	3.3	3.3
1:4	1:4	9	8	2	2
1:4	1:4	8	8	2	2
1:2	1:4	dead	8	-	2
	1:4		7		2
	1.4		5		2
OTALS				10.6	16.6

Legend - Table 2

The data presented here is an average value for colony formation for 8 wells. Each well was seeded with 50,000 cells at PDL 20 into 0.33% agar over a 2.0% agar base. The colonies were counted after 21 days and the frequency of colony growth normalized to 100,000 cells per well.

Table 2. Frequency of Colony Growth in 0.33% Agar Overlay Over a 2.0% Agar Base.

Exp . No.	PDL	Growth in Soft Agar
1	20	20.0
2	20	.15.0
3	20	25.L

PRESENSITIZATION OF HUMAN CELLS WITH

EXTRINSIC SIGNALS TO INDUCED CHEMICAL CARCINOGENESIS

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Running Title: Modulation of Early Stages of Carcinogenesis

Footnote:

Abbreviations

Population doubling, PDL; fetal bovine serum, FBS; complete medium, CM; hydrocortisone, HC; 17-β-estradiol, E_2; progesterone, P; cortisone, C; Dulbecco's modified minimum essential medium, DM; Minimum essential medium, MEM; Specific activity, S.A.; 9-octadecenoic acid (oleic acid), C 18:1; arachidonic acid (eicosatet-raenoic acid-5,8,11,14), C 20:4; dibutyryl cyclic GMP, d-cGMP; insulin, IN; phorbolmy-ristate acetate, PMA; anthralin, Anth; N-methyl-N'-nitrosoguanidine, MNNG; human nasopharyngeal carcinoma cell line, NPC; SV-40 transformed WI-26, VA-4; Eagles Basal Medium, EBME; Low Calcium - Eagles Basal Medium medium, LoCal-EBME.

Summary

Foreskin derived low passage human cell populations were reproducibly transformed with chemical carcinogens when the cells were blocked in G_1, released from the block, and treated with either the carcinogen N-methyl-N-nitrosoguanidine (MNNG) or with Aflatoxin B1 in the "S" period of the cell cycle. Arginine and glutamine deficient medium was required to effectively block the cells in the G_1 period. Estradiol, insulin, anthralin or phorbol myristate acetate sensitized the cell population to carcinogen treatment when added 10 hr prior to the addition of the carcinogen in early "S" period. Presensitized cells kept blocked in G_1 period for 48 hr or longer, released and treated in "S" period with MNNG or Aflatoxin B1 were not transformed; nor did transformation occur in presensitized cell populations treated in G_2 (4.5 hrs), M (1.5 hrs) or G_1 (8.2 hrs). Cells derived from carcinogen-treated presensitized cells grew as colonies in soft agar at 16-20 PDL. When cells derived from colonies isolated from the soft agar were injected subcutaneously into nude mice tumors developed.

INTRODUCTION

Serially subpassaged human cells grow in vitro as randomly proliferating monolayer cultures with subpopulations capable of different rates of scheduled (Cristofalo and Sharf, 1973) and/or unscheduled DNA repair synthesis (Hart and Setlow, 1976). With continued subpassaging the non-proliferating populations constitute a progressively larger proportion of the total cell population. These changes in cell cycle kinetics with serial subpassaging could be due to a decrease in proliferating subpopulations (Cristofalo and Sharf, 1973; Merz and Ross, 1973; Turk and Milo, 1974; Milo and Hart, 1976) or to a lengthening of the G_1 or G_0 cell cycle phase (Grove and Cristofalo, 1977). In the terminal passages of Phase III unscheduled DNA repair synthesis also decreases in subcultured human diploid populations (Milo and Hart, 1976).

The expression of the transformed phenotype after a carcinogenic insult requires preferential cell multiplication. The failure of cell proliferation and fixation of the initial transformation event results in the chemically treated cells becoming part of the cell population belonging to a permanent resting phase of the cell cycle. In this way prevention or suppression of cell transformation would occur.

Previously we demonstrated that chemical carcinogen-induced transformation of human cells occurs in low passage populations that are first blocked in G_1, released from the block, and treated in "S" (Milo and DiPaolo, 1978). The current study shows that several chemicals may sensitize cells and alter the subsequent transformation response of human cells to chemical carcinogens.

MATERIALS AND METHODS

Cell Cultures

Neonatal human foreskin cell suspensions obtained by collagenase dispersion (Riegner et al., 1976), were seeded into 25-72 cm^2 flasks and produced confluent monolayers within 48 hrs. These cell cultures have a finite replicative capability of 35 $\pm$ 7 population doubling (PDL). Cultures containing rapidly proliferating cells (Cristofalo and Sharf, 1973) were arbitrarily assigned level 2 after the first subpassage. All populations were routinely passaged on Eagles' Minimum Essential Medium supplemented with non-essential amino-acids, sodium pyruvate, gentocin, glutamine. 25 mM Hepes at pH 7.2 (Milo and DiPaolo, 1978) and 10% fetal bovine serum (FBS). All FBS used in the above complete medium (CM) for these experiments was first analyzed for content of hydrocortisone (HC), 17-β-estradiol (E$_2$), progesterone (P), cortisone (C) (Milo et al., 1976), and unsaturated fatty acids (Huttner et al., 1978). FBS selected for the carcinogenesis-synchronization experiments optimally exhibited growth potential of 40-50 % colony forming efficiency in cultures seeded at 250 cells/25 cm^2 flask.

G$_1$ Period

To block the human cells in the G$_1$ period several variations of Dulbecco's Modified Minimum Essential Medium (DM) deficient in specific amino-acids were used: lacking either leucine-glutamine, isoleucine-glutamine, leucine-arginine, isoleucine-arginine, or glutamine-arginine combinations, or one of the individual amino acids. The technique used to block the cells in the "G$_1$" period was modified from the Tobey and Ley procedure (1971). Cell suspensions of 5000 cells-cm^{-2} were seeded onto 4 microscope slides and placed in 176 cm^2 petri dishes containing 50 ml of each amino acid deficient DM medium supplemented with dialyzed 10% FBS (d-FBS). Cells were

incubated in a 4% CO_2-enriched air atmosphere at 37°C. The cultures were released from the "G_1" period block by exchanging the amino acid deficient medium to growth medium (CM) consisting of Eagles' MEM 10% FBS, with the addition of ^{3}H-thymidine (1 µCi/5ml) and, where appropriate to the experiment, an extrinsic factor such as IN. The radiolabeling period for continuously labeled cells was 96-144 hr (S.A. 6.0 Ci/m mole of ^{3}H-thymidine) or for pulse labeled cells, 30 minutes (S.A. 60 Ci/m mole of ^{3}H-thymidine). The CM, including radiolabeled thymidine, was replaced every 24 hr in the continuously labeled culture. After the block was removed, chemicals known to alter cellular DNA synthesis were suspended in CM and added to the cell population at either 24, 48, 72, 96 or 120 hr.

Chemicals

The extrinsic factors studied for their effect on the "S" phase of the cell cycle were oleic acid (9-octadecenoic acid (C18:1); arachidonic acid (eicosatetraenoic acid - 5, 8, 11, 14 (C20:4) (Nu-Chek, Elyrian, Minn.; Huttner et al., 1978) at 5 µg/ml; dibutyryl cyclic GMP (d-cGMP) at 2.07 µg/ml; insulin (IN) at 0.5 U/ml: 17-β-estradiol (E_2) at 1 µg/ml; hydrocortisone (HC) (Schwartz/Mann, Orangeburg, NY) at 1 µg/ml; phorbol-myristate acetate (PMA) (Consolidated Midland Corp., Brewster, NY) at 1 µg/ml; and anthralin (Anth) (Pfaltz and Bauer, Inc., Stamford, Conn.). Chemicals were dissolved in acetone (Spectrar Grade, Mallincrodt, Inc.) under red light in an argon atmosphere, and maintained in stock solutions of 1 mg/ml. The carcinogens, aflatoxin B_1 and N-methyl-N'-nitrosoguanidine (MNNG), were dissolved in acetone and stored at -19°C until immediately before use. Final culture concentration of acetone was 0.02%. Cell cultures that had been pulsed with ^{3}H-thymidine (60 Ci/m mole) for 30 minutes, or continuously (6.0 Ci/m mole) for 120 hr, were acid washed with 0.1 N HCl to remove free ^{3}H-thymidine. The culture slides were fixed in Carnoys' solution or

methanol: acetic acid (3:1, v/v), dipped in Kodak NTB-2 emulsion, exposed for 3-4 days at $12^{\circ}C$, developed in Kodak D-19 developer, fixed in Kodak fixer 197-1746, and stained with filtered Giemsa.

Preparation of Metaphase Chromosomes

Rapidly proliferating cell cultures at PDL 1-5 were prelabeled with 1 μCi/ml ^{3}H-thymidine (6.0 Ci/m mole) for 24 hr. Grains were found on 100% of the interphase nuclei. Companion cultures radiolabeled in the same manner were fed every 24 hr with amino acid deficient DM (pH 7.2) supplemented with 10% d-FBS. Sixteen to 24 hr later the deficient medium was replaced with CM; 0.1 μg/ml colcemide was added three hr prior to fixation. Samples were removed every 30 minutes beginning four hr into the S phase, fixed in Carnoy's solution and either stained with aceto-orcein before dipped in Kodak NTB-2 emulsion or post-stained with Giemsa following development (Baserga, 1967) four days later in Kodak D-19 developer and fixation in Kodak fixer 197-1746.

Growth in Soft Agar

Two transformed cell lines were used as positive controls for evaluating the growth potential of chemical carcinogen-treated cells in soft agar. These were a human nasopharyngeal carcinoma cell line (NPC; gift from Litton Bionetics, Kensington, MD) and a SV-40 transformed lung cell line (VA-4; from the A.T.C.C., Rockville, MD). Both grew in soft agar (0.35% containing EBME-LoCal supplemented with 20% FBS) over a 1% or 2% agar base containing RPMI 1629 growth medium supplemented with 20% FBS (Milo and DiPaolo, 1978). The frequency of bolus formation with NPC and VA-4 after 9-11 days was 70-80% and 80-85%, respectively. The chemically treated cells were seeded at 50,000 cells-25 cm^{-2} well (Milo and DiPaolo, 1978) and examined 11 days later.

Growth in Nude Mice

Nude mice were purchased from Sprague-Dawley (Madison, Wisc.) and delivered at 6 weeks of age. Mice between 10-12 weeks of age, previously subjected to 450 R whole body irradiation, were inoculated subcutaneously with 10^6-$10^{7.5}$ NPC cells-inoculum^{-1} into the subscapular region. After 24 hrs the initial bleb regressed and 4-6 weeks later a tumor 0.6-1.2 cm in size was excised. The optimum inoculum size was 5 x 10^6 cells. VA-4 cell inocula regressed in 24 hr and no visible tumor was seen in the 20 inoculated mice after 6 month period. The tumor incidence in NPC-inoculated mice was 5/10. The chemical carcinogen-treated cell populations were inoculated at a cell inoculum size of 5 x 10^6 cells in 0.25 ml volume in the manner previously described and submitted for pathology (Milo and DiPaolo, 1978). After 4-6 weeks the tumors were removed and submitted for pathology.

RESULTS

Cell Block

Cells from amino acid-deficient DM preparations supplemented with 10% d-FBS contained varying numbers of radiolabeled nuclei (Fig 1). At 96 hr cells maintained in minus glutamine and arginine (Medium A), minus leucine and glutamine (Medium or minus isoleucine and glutamine (Medium C) contained 5, 37 and 57% radiolabeled phase cells, respectively.

In DM deficient media lacking only one amino acid (i.e., arginine, glutamine, or isoleucine) the cell populations contained 50-70% radiolabeled interphases a 96 hr period. A 65% increase in cell numbers 10 hrs after the S period ponded to the number of radiolabeled interphase nuclei observed.

Medium A was selected for blocking the cells in G_1, and samples were checked at two-hr intervals. Cell populations seeded for 24 hr in medium A and transferred to CM for 72 hr contained 67-72% radiolabeled nuclei. Cells kept in medium A for 48 hr prior to transfer to CM contained 35-42% radiolabeled nuclei at the end of an additional 72 hr period. The area in Fig. 2 between the curves for randomly proliferating cell populations (open circles) and the cells held in medium A for 24 hr (filled circles) represents cells that respond to IN, Anth, E_2, or PMA treatment (see below). The area described by the cell population that was held in medium A for 48 hr (Fig. 2, triangles) represents a population that will not totally respond to added signals such as IN, Anth, or E_2.

Effect of Exogenous Factors on Cell Growth

Cell populations were transferred from medium A to CM after 24 hr and one of the following chemicals was added: IN, HC, C20:4, E_2, d-cGMP, PMA, or Anth (Fig. 3), along with ^{3}H-thymidine. In these experiments, untreated control cultures transferred to CM after 24 hr in medium A contained radiolabel in only 62-67% of cell nuclei up to 120 hr after transfer. Either IN, C20:4, E_2, d-cGMP or HC added to CM amplified the appearance of radiolabeled nuclei over the 120 hr sampling period. Anth or PMA did not alter the profile of ^{3}H-thymidine incorporation into nuclei. IN-treated cells recovered from the block more rapidly than any of the other cultures. Whenever treated cultures were removed from the experimental medium and passaged, the normal proliferative kinetics resumed by passage 2 or 4. The PDL time after a 1:2 split was approximately 2-3 days at PDL 2. In no case was the lifespan of cultures appreciably altered from 37 ± 7 PDL.

To further study the effect of these compounds on the proliferative capability of the cells, populations were maintained in medium A for 48 hr (Fig. 4) before

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treatment. Untreated control cells in these experiments contained 43% radiolabeled nuclei 48 hr after transfer from medium A. IN-treated cell populations contained 72% radiolabeled nuclei. C20:4- or HC-treated cultures sampled for up to 120 hr incorporated ^{3}H-thymidine into no more than the 72% of nuclei labeled at 48 hrs. Again, in Anty-or PMA-treated cultures the labeling profile did not differ from the untreated cultures.

Since it was difficult to predict the exact length of the lag interval before the cells in G_1 would begin to enter the "S" period following transfer from medium A to CM, the cells were blocked in G_1 in medium A for 24 hr and then released. At point B (Fig. 5) either IN or one of the other compounds (See Figs. 1-3) were added to the CM. At 30 minute intervals for the next 25 hr, sample populations on 1 mm were removed and placed in CM containing 1.0 μCi of ^{3}H-thymidine (60 Ci/m mole) in 5 ml for 30 minutes. Untreated control cultures achieved a level of only 73% radiolabeled nuclei, whereas cells treated with IN reached a 90-95% level.

When E_2, Anth or d-cGMP (Table 1) were added to pulse labeled cultures, the "S" peak occurred 1 hr later. Compared to untreated controls, the length of S (i.e., the appearance and disappearance of labeled interphase nuclei) did not vary in any of the treated populations; the length of "S" in all populations was 8.2 hr. The length of M, 1.5 hr, was determined by measuring the interval for the appearance and disappearance of radiolabeled metaphase nuclei. The interval between "S" and M (G_2) was calculated to be 4.5 hr. The length of the cell cycle, 22.4 hr, was determined by counting cells over a 24 hr period using trypan blue dye exclusion. Subtraction of the experimentally determined "S" and M times and the calculated time for G_2 from 22.4 yielded an estimated G_1 of 8.2 hr. If either IN or Anth was added at interval B (Fig. 5) the period most dramatically affected was G_1. G was shortened from 8.2 to 6.5 hr between

waves of cells passing through "S", G_2 and M for three cycles. After the first cycle and for each successive cycle 20-22% of the cells departed from the synchrony pattern. After the second cycle the number of rapidly pulse-labeled nuclei decayed to that of randomly proliferating companion cultures. The absolute values for the total number of cells in "S" varied with tissue and according to treatment. When experimental sister cultures were treated with PMA, IN, Anth or E_2 at point B (Fig. 5) the number of cells in "S" varied from 67-95% (Table 1).

Cell Transformation Studies

Ten hr after administration of IN or a compound listed in Table 1 at interval B (as the cells were entering "S"; Fig. 5), aflatoxin B_1 or MNNG was added to the cultures. The carcinogens were removed 12 hr later and the cultures serially passaged into CM containing 8X non-essential amino acids and 2X vitamins (Milo and DiPaolo, 1978), for 15 PDL. The populations were then serially passaged into soft agar (0.35%, containing EBME-LoCal, supplemented with 20% FBS) over a 2% agar base containing RPMI 1629 growth medium supplemented with 20% FBS (Milo and DiPaolo, 1978). After 11 days colonies were scored, removed and serially subpassaged in CM. Growth in soft agar for treated cultures ranged from no growth to $1:10^{2.5}$ for aflatoxin B_1-anthralin treated cultures (Table 2). Cell populations treated with non-carcinogenic compounds and passaged through soft agar sometimes formed 2-6 cell short chains rather than a colony. None of these cells formed colonies in agar when passaged a second time. Addition of IN, E_2, PMA or Anth at interval B enhanced colony formation in soft agar as compared with aflatoxin treatment alone. Serially passaging the cell a second time in the agar increased the frequency of colony formation to 40-90%. Similar results recurred with MNNG-treated cell populations regardless of whether IN or Anth were used (data not shown here). PMA-aflatoxin B_1 treated

populations, while exhibiting a low frequency of colony formation upon passage through the soft agar for the first time, did form colonies of 50-300 cells per colony, followed by an increase in frequency in colony formation to 70% during the second agar passage.

Addition of oleic acid (C18:1), C20:4, or d-cGMP did not enhance colony formation. E_2 and PMA were intermediate in their effect on colony formation by carcinogen-treated cells, while IN or Anth were quite effective in augmenting colony formation (Table 2). After carcinogen-treated cells isolated from agar were serially passaged, 5×10^6 cells were inoculated subcutaneously into the subscapular region of previously irradiated (450R whole body) nude mice (Milo and DiPaolo, 1978). Subcutaneous tumors appeared at the injection site within 10-18 days. The tumors were scored 4 weeks later when 0.8-1.5 cm in size. Cell populations treated with aflatoxin B_1-IN or Anth, or with MNNG-IN or Anth, produced the highest numbers of tumors (Table 3). In three experiments (data shown for one experiment in Table 3) the order of successes was the same. Aflatoxin B_1-IN or MNNG-IN induction of tumors was the highest, followed by Aflatoxin B_1-Anth, or -PMA. Tumors excised and examined histopathologically and karyologically were all confirmed as undifferentiated mesenchynal tumors of human origin. To date, all cell lines that have been grown in soft agar, and successfully serially passaged a second time in soft agar, have produced tumors when injected in nude mice. No cell populations after being blocked for 48 or 72 hr and treated in "S" with Aflatoxin B_1-IN or MNNG-IN formed colonies in soft agar or produce tumors in nude mice.

DISCUSSION

As previously reported (Milo and DiPaolo, 1978), we have successfully induced transformation of normal human cells with different chemical carcinogens. Other

reports suggest that randomly proliferating normal skin cell populations can be transformed by 4-nitroquinoline oxide and MNNG. However, we have found that successful induction of neoplastic transformation in rapidly proliferating normal human foreskin cells by chemical carcinogens in vitro is an exceedingly rare event. Chemicals that act as carcinogens in neonatal foreskin cells in vitro can damage cellular DNA (Milo et al., 1978), but the DNA repair systems in these normal diploid cell populations are extremely rapid and error-free (Maher et al., 1977).

Randomly proliferating normal cell populations repair over 90% of the damage from chemical carcinogens in 4-10 hr (Milo and Hart, 1976) and are rarely transformed. There are two methods for synchronizing mammalian cells in G_1 phase of the cell cycle; one is to arrest the cells in confluence and the other is amino acid deprivation (Peterson et al., 1974; Jones et al., 1975; Jones et al., 1977; Grisham et al., 1979). Following release from the block nearly all the cells enter "S" phase within 6 to 8 hr. C_3H 10T1/2 cells arrested in this manner, released and treated with MNNG in "S" phase exhibit an increase in sensitivity to MNNG and become more readily transformed. However, isoleucine deficient medium does not block human cells in G_1. Furthermore, release of human cells from block by subpassaging the cells, after density dependent inhibition in G_1 with carcinogens over a 10 hr period, does not result in a carcinogen-induced transformation event. In fact, the transformation of human cells at early passage levels is inhibited if the transformable cells are kept in a density-inhibited state for 8-16 hr prior to subpassaging. Moreover, cultures held in the G_1 phase of the cell cycle for more than 24 hr cannot be transformed when treated with aflatoxin B_1 or MNNG in the "S" phase. A double amino acid deficient medium (minus glutamine and arginine) was necessary to adequately block the cells in G_1. Augmentation of the transformation occurs when IN, Anth, E_2, or PMA are added to

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the cultures prior to carcinogen administration. The major difference between the effectiveness of aflatoxin B and MNNG as carcinogens appears to be the time at which the compounds are added to the cells entering "S". MNNG is more effective if added early in "S" (0-4 hr after recovery from the amino acid block) while aflatoxin B_1 is more effective if added from 2 hr prior to "S" to 4 hr into "S". The incidence of transformation was reduced to less than 1 out of 10 when these compounds were added to the cells in late "S" (4 to 8 hr into S)..

IN, PMA, E_2, and Anth have been found to modulate the expression of other transforming agents, and the effects on chemical carcinogen-induced carcinogenesis are not unique. Enhancement of virus-induced transformation by some of the same chemical factors used here occur with E_2 (Milo et al., 1972), HC (Schaller et al., 1976), and PMA (Weinstein et al., 1979), these chemicals can interfere with semi-conservative DNA synthesis (Milo and Hart, 1975). Modulation of cellular functions is not an unusual response by cells to the presence of these compounds and they obviously facilitate transformation by chemical carcinogens. This suggests that, as in the 3T3 system described by Grisham et al. (1979), that human cell subpopulations are sensitized in "S" to carcinogens by amino acid deprivation and reconstitution. This sensitization is enhanced by Anth, IN, PMA or E_2 at pharmacological concentrations of the drugs.

Many of the compounds studied here (E_2, HC, C20:3, C20:4, IN) are components of fetal bovine serum and serve as natural regulatory agents of proliferation, the action of PMA has been suggested to resemble that of hormonal agents. The pleiotypic responses of subpopulations to the carcinogen treatment may be induced by pretreatment with PMA, E_2, Anth or IN. Therefore, after subsets of populations have

been modulated by being kept in G_1 for 48 hr or serially subpassaged in vitro to PDL >5, the cells no longer respond to the extrinsic factors in the presence of the carcinogens.

ACKNOWLEDGEMENTS

TABLE 1

PERCENT ^{3}H-THYMIDINE LABELED NUCLEI DURING THE PEAK TIME INTERVAL OF SCHEDULED DNA SYNTHESIS OF CELL POPULATION TREATMENT WITH DIFFERENT EXTRINSIC FACTORS.

				Treatment				
Time (hr).	C20:4	IN	HC	E_2	Anth	PMA	d-cGMP	Untreated
34	35	42	45	21	22	17	12	11
35	74	77	74	33	33	23	47	49
36	90	92	89	51	41	35	67	72
37	84	81	81	91	55	33	72	67
38	63	72	72	67	38	15	63	54
39	57	60	57	37	22	7	55	34

Concentrations used were below level that inhibited cellular proliferation as evaluated by relative plating efficiency of the cell population (Milo et al., 1976).

Legend for Table 2

The carcinogenic activity of Aflotoxin B1 in cultures in the presence of added factors that altered the response pattern are presented. The exogenous factors selected for this study were insulin, (IN); estradiol, (E_2); arachidonic acid, (C20:4); oleic acid (18:1); defutyryl cyclic GMP, (d-cGMP); phorbol myristate acetate, (MPA); and anthralin, (Anth). Each of these factors was added at point B as described in Fig. 5. Ten hr later as the cells were entering "S" aflatoxin B_1 was added to the cells. After the treated cells had passed through "S" both the cultures treated with the factor alone and/or carcinogen and factor were removed (18.3 hr) after point B (Fig 1). Column 1 presents the concentration of the carcinogen and factor added to the cells. Column 2 presents the number of lines that grew in soft agar/number of lines seeded in soft agar. Column 3 presents the frequency obtained per number lines that grew in soft agar. Frequency is the number of colonies formed in 21 days per 50,000 cells seeded into at 0.35% agar overlay (Milo and DiPaolo, 1978). The results were expressed as log values to the base 10.

TABLE 2

FREQUENCY OF COLONY GROWTH IN SOFT AGAR OF TRANSFORMED HUMAN CELLS TREATED WITH DIFFERENT EXTRINSIC FACTORS AND AFLA-TOXIN B_1.

Chemical (μg/ml)	No. of Treated Populations	No. of lines that grew in S.A./ No. of lines seeded in S.A.	Frequency
Control (0)	0	0	0
Aflatoxin-B_1 (10)	10	3/20	$1{:}10^{5.2}$
Aflatoxin-B_1 (10) - IN (1)	10	10/10	$1{:}10^{3.1}$
IN (1)	3	2/3	$1{:}10^{5}$
Alfatoxin-B_1 (10) E_2 (1)	2	2/2	$1{:}10^{4.0}$
E_2	2	1/2	$1{:}10^{5.2}$
Aflatoxin-B_1 (10) C20:4 (1)	3	3/3	$1{:}10^{5.2}$
C20:4 (1)	3	1/3	$1{:}10^{5.4}$
Aflatoxin-B_1 (10) C18:1 (1)	3	1/3	$1{:}10^{5.7}$
C18:1 (1)	3	0/3	0
Aflatoxin-B_1 (10) d-cGMP (1)	2	1/2	0
d-cGMP (1)	2	0/2	0
Aflatoxin-B_1 (10) PMA (1)	5	5/5	$1{:}10^{4.7}$
PMA (1)	3	0/3	0
Aflatoxin-Anth (1)	4	4/4	$1{:}10^{2.5}$
Anth (1)	3	1/3	$1{:}10^{5.2}$

TABLE 3

EFFECT OF EXTRINSIC FACTORS ON GROWTH OF TRANSFORMED CELLS IN SOFT AGAR AND TUMOR FORMATION IN THE NUDE MOUSE.

Treated Cell Populations	Extrinsic Factors	*Frequency of Growth in Soft Agar	*Growth in Nude Mouse	**No. of successes/ No. of attempts
Aflatoxin B_1	none	+	+	1/8
	IN	+	+	7/7
	Anth	+	+	5/7
	PMA	+	+	2/8
	E_2	+	+	4/11
	C20:4	-	-	
	C18:1	-	-	
	HC	-	-	
	d-cGMP	-	-	
None	none	-	-	
MNNG	none	+	+	1/11
	IN	+	+	4/5
	Anth	+	+	5/5
	PMA	+	+	3/9
	E_2	+	+	2/8
	C20:4	-	-	
	C18:1	-	-	
	HC	-	-	
	d-cGMP	-	-	
None	none	-	-	

*The dashes (-) indicate there were no observable responses (bolus formation of less than 50 cells) within 14-21 days in soft agar and no response within 6 months for growth of the cells isolated from soft agar. Transformed cells were injected at a cell concentration of 5×10^6 cells/0.5 ml into the subscapular region of the nude mouse. The tumors were allowed to grow to 0.6-1.2 cm in size (Milo and DiPaolo, 1978). Soft agar is defined as a 0.35% upper layer of agar containing growth medium on a 1% or 2% agar base (see text for details). Control (untreated cell) inocula did not grow in soft agar and when injected directly from culture $(10^7$ cell-inoculum$)^{-1}$ into the mouse the bleb(s) regressed in 24 hr.

Legend - Figure 1

Human foreskin cell populations were seeded at a cell density of 5,000 cells-cm^{-2} onto a 15 mm diameter microscope slide or a coverslip. These seeded slides were then placed into 25 cm^2 dishes containing 5 ml of DM supplemented with 10% d-FBS minus arginine-glutamine (•—•), minus leucine-glutamine, (▲--▲), or minus isoleucine-glutamine (o—o), and monitored for the presence of radiolabeled interphases for 96 hr after seeding. The deficient medium was replaced every 24 hr. ^{3}H-thymidine (1 Ci/5ml; S.A. 6 Ci/m mole) was added at the time the cells were seeded into the DM. The radiolabel was replaced every time the DM or CM was replaced. Four samples were taken at 1 hr over the 96 hr period, fixed and prepared for autoradiography (see text).

fig 1
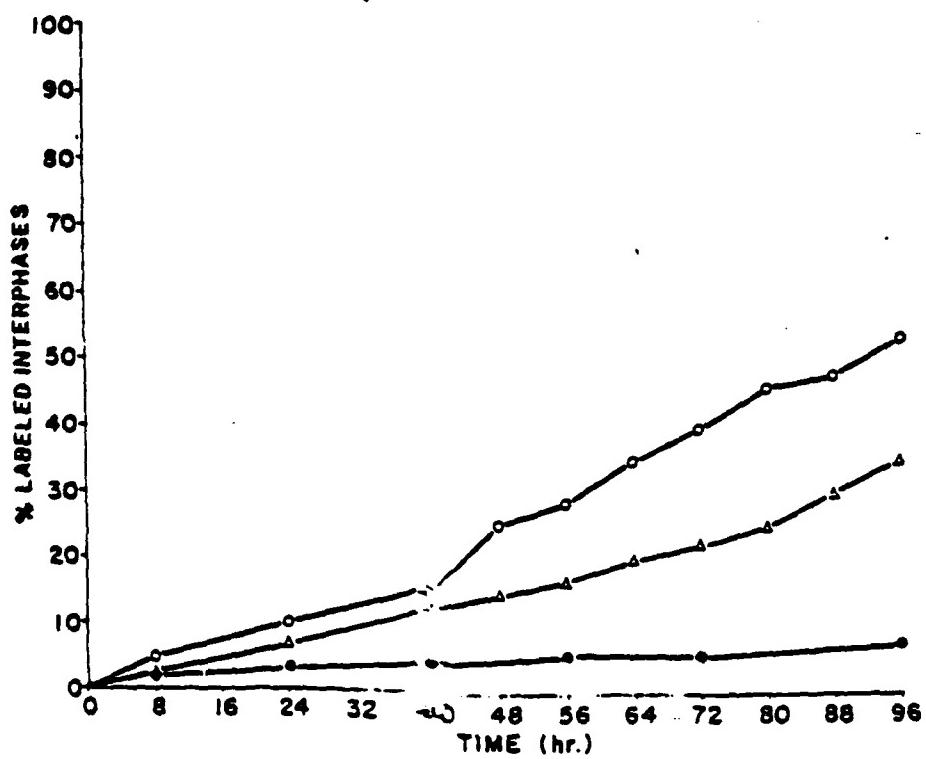
Legend - Figure 2

Human foreskin cell populations at saturation density were seeded at a cell density of 5000 cells-cm^{-2}. These populations were not permitted to remain in saturation density for more than 16 hr prior to seeding. The cells were suspended by trypsinization (Riegner et al., 1976) and the cells recovered by centrifugation at 650 X g. The pellet was resuspended into experimental medium A and seeded onto 15 mm diameter microscope sides or coverslips for 6 hr to attach to the substratum. These cultures were kept in this deficient medium for 24 hr (●—●) (see Legend 1) or 48 hr (Δ—Δ). The black was removed by the addition of 10% FBS-supplemented CM containing 2 mM arginine and 1 mM glutamine. One micro curie of ^{3}H-thymidine (6.0 Ci/m mole)/5 ml was added to each amino acid deficient experimental medium and each complete growth medium. In each case both medium and radiolabel thymidine were replaced at each and every 24 hr period from seeding to completion of the experiment. Cells kept continuously in CM and serially subpassaged into CM were sampled every 2 hr from 0 hr up to 96 hr (o—o) post-seeding.

Proliferating cell populations seeded into experimental medium A for 24 hr (●—●), 4 samples were removed every 2 hr from 0 hr (at 24 hr time pt) up to 96 hr. The block was removed at 24 hr. Additional populations were left in experimental medium up to 48 hr (Δ—Δ) the block removed. Another population was left in the amino acid-deficient medium from 0 hr up to 120 hr (▲—▲). The medium plus radiolabel thymidine was replaced every 24 hr.

Fig 2
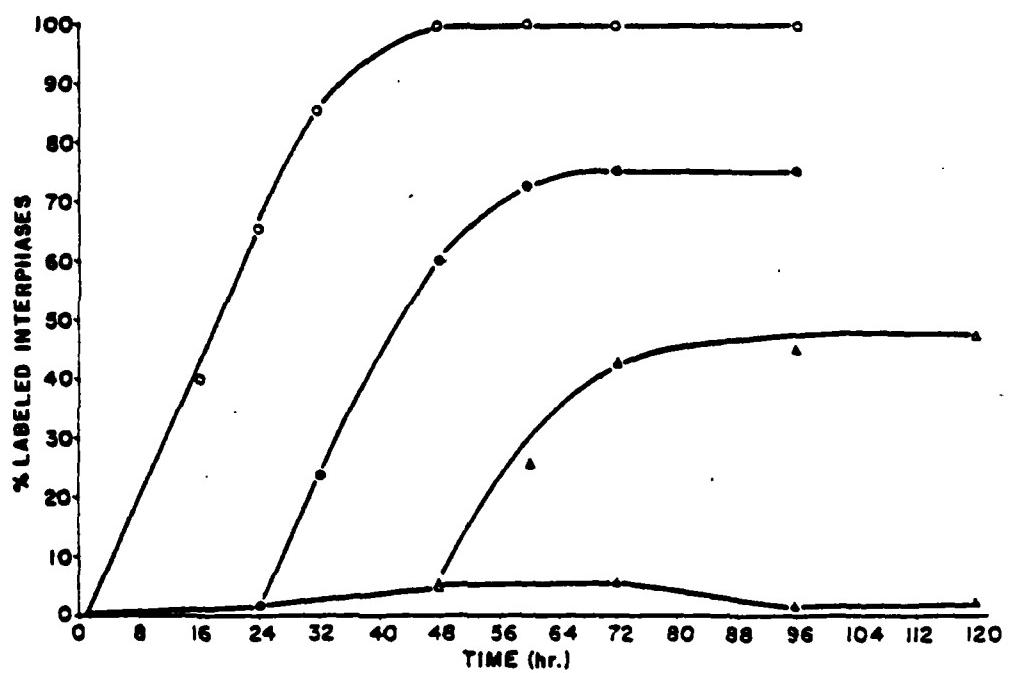
Legend - Figure 3

Randomly proliferating cell populations that were not in saturation density arrest for more than 16 hr were seeded into CM (o—o) or medium A (■—■) and allowed to attach for 6 hr ($\geq$95% attachment was determined by fixation, staining and counting the number of cells that attached to the substratum versus the number of cells seeded). All populations seeded into medium A were transferred to CM plus 2 mM arginine and 1 mM glutamine and either HC, C20:4, E_2 (▲—▲); or IN (△—△); or PMA (●—●); or Anth (●—●); or d-cGMP (□—□) added to each separate culture at 24 hr. Radiolabel thymidine was added to the cultures as described in the legend for Fig. 2. Cultures were refed every 24 hr over the 120 hr period.

Fig 3
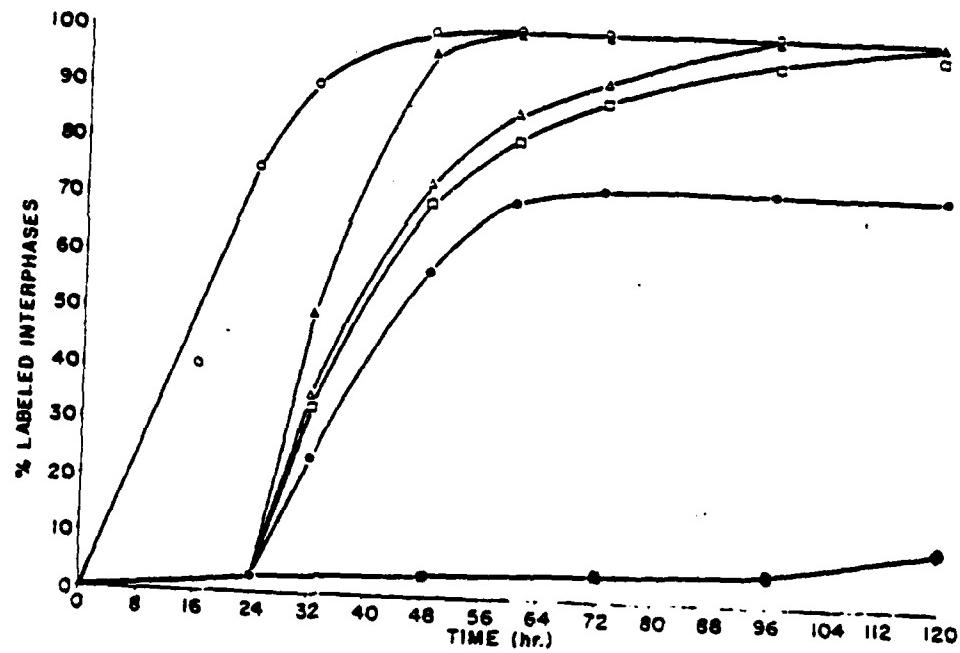
Legend - Figure 4

Cell populations used in these experiments were prepared and seeded in the same
manner as described in the legend for Fig. 3. However, these cultures were released
from block after 48 hr. Radiolabel thymidine was added to medium A and/or CM as
decribed in the legend for Fig. 2. Control cultures (●—●) were released after 48 hr and
IN (o—o) or HC (▲—▲) added. Other compounds listed in the legend of Fig. 3 induced
responses intermediate between IN (o—o) or HC (▲—▲) treated cultures. These
compounds were added to CM at 48 hr after replacement of medium A. The untreated
control cultures were left in medium A (Δ—Δ). Radiolabel thymidine was added to
the cultures at seeding and every 24 hr upon replacement of medium A or CM.

Fig 4
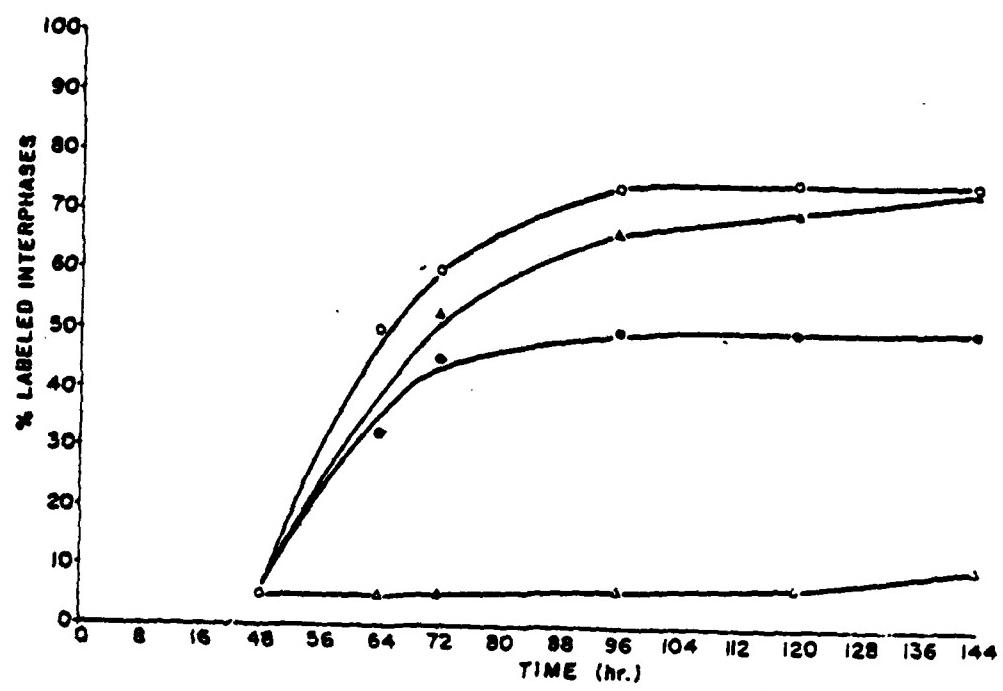
Legend - Figure 5

Cell populations were seeded at a cell density of 5,000 cells-cm^{-2} with DM minus arginine-glutamine, (A) supplemented with 10% d-FBS. The cells were fixed 24 hr later, stained with hematoxylin, and enumerated (continuously 95% absolute plating efficiency occurs). The DM was removed at this time and the cultures released with CM (see text) containing 0.5 U of insulin per ml. Companion wells containing 5 ml of CM with ^{3}H-thymidine were incubated under identical culture conditions as for the experimental DM cultures. One microcurie of ^{3}H-thymidine (60 Ci/m mole^{-1}) was added. At 30 minute intervals samples were removed from DM or CM medium, incubated for 30 minutes in the radiolabeled CM medium, fixed, stained and developed under NTB-2 emulsion for 4 days. The labeled interphases were enumerated. Companion slides and labeling conditions were used in controls except 0.1 ug/ml of colemide was added to the CM medium. Four hrs into "S", samples were taken and radiolabeled for 30 minutes in CM, fixed, and developed under NTB-2 emulsion (see text). Radiolabeled metaphases were enumerated and the percentages compared to the controls were calculated (not reported here).

Fig.5
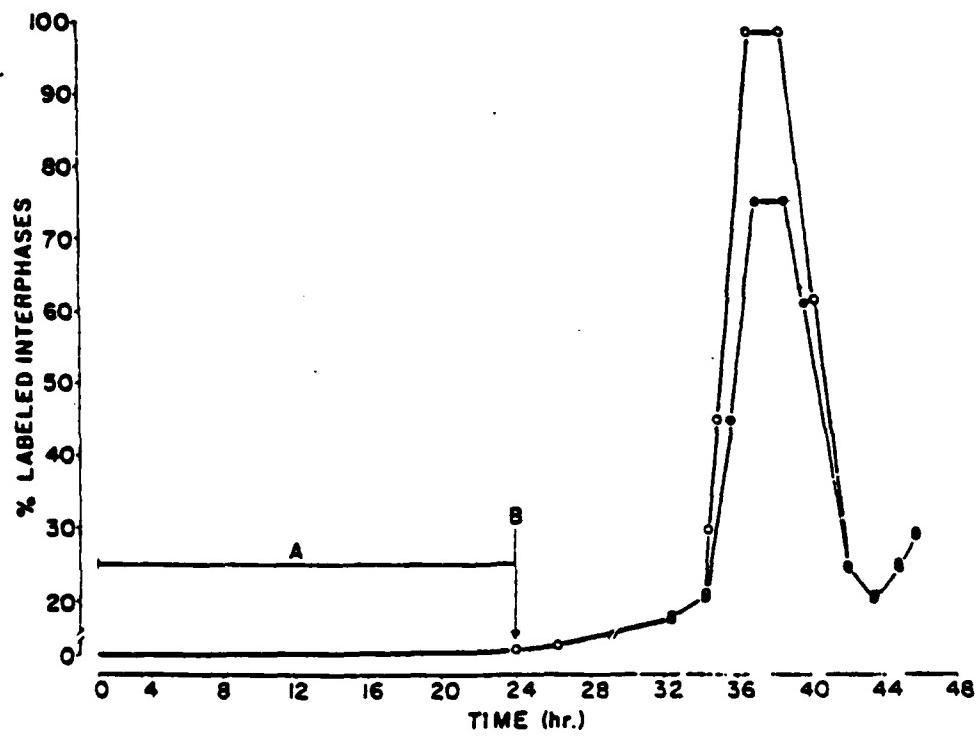
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Segment II

Work Scope - 1979

I. Original Objectives

1. Determine whether suspect fuels with carcinogenic potential inhibit or enhance ST-FeSV virus-directed transformation of human cells in a predictable manner and correlate effects on virus transformation with _in vitro_ chemical transformation in Segment I.

2. Investigate activation of endogenous human virus by suspect carcinogens.

3. Investigate hormonal influence on chemical-viral interactions.

4. Develop techniques and procedures to be used in hybridization analysis of chemically treated ST-FeSV virus-infected cells.

Specific Objectives of Segment II

1. Determine whether napthylamines, hydrazine (HZ), monomethylhydrazine (MMH), unsymmetrical dimethylhydrazine (UDMH) and symmetrical dimethylhydrazine (SDMH) enhance or inhibit Snyder-Theilen Feline Sarcoma Virus directed cell transformation of human skin cells _in vitro_ in a predictable manner.

2. Determine whether HZ, MMH, UDMH and SDMH activate endogenous C-type RNA virus expression in human cells.

3. Develop techniques, procedures and materials for hybridization analysis of HZ, MMH, UDMH, SDMH and BP treated ST-FeSV infected human cells for increased or decreased genome expression.

II. Progress to Date

1. Determined alpha-napthylamine (ANA), phenyl-alpha-napthylamine (PANA)
   and phenyl-beta-napthylamine (PBNA) significantly inhibited ST-FeSV
   transformation of human skin fibroblasts.

2. Determined HZ, MMH, UDMH and SDMH inhibited ST-FeSV transformation.
   Inhibition was dependent upon temporal relationship of treatment to
   infection.

3. Results of co-carcinogenesis assays showed high degree of correlation
   with _in vitro_ chemical transformation and neoplastic transformation
   in Segment I.

4. Determined long-term exposure of human skin fibroblasts (HSF) to HZ,
   UDMH, MMH (FY 78) and SDMH (FY 79) did not activate human endogenous
   virus.

5. Determined LD50 for shale oil and petroleum derived JP5 and DFM fuels.

III. Introduction and Background

Hydrazine, hydrazine derivatives, and polycyclic hydrocarbons are
of increasing biological concern because of their potential to act as
mutagens or carcinogens. Thus, efforts must be concentrated toward de-
veloping rapid, sensitive and inexpensive biological assays which would
determine carcinogenic and mutagenic potential of these compounds.

While many biologic effects of hydrazine fuels and its derivatives
have been studied in animals, the extrapolation of these biological effects
to man has been difficult because of differential responses manifested in
diverse species of test animals. Prolonged exposure of test animals to
these compounds has suggested potential tumorigenic and carcinogenic
activity with a diverse number of tumor types noted, depending upon the
species and fuel component used. For example, Diwan et al. (1) concluded

that genetic differences with inbred strains of mice affected the response
of DMH carcinogenesis. Thus, carcinogenesis assays in rodents may lead
to false negative results based on that genetic strain used in the assay.

Rats, mice (2,3), syrian hamsters (4) either fed or injected with HZ
and its derivatives, developed different types of tumors or leukemias
depending upon the route of administration, the compound used and the
species involved in the assay. Pulmonary tumors, reticulum cell sarcomas,
myelogenous leukemia and histocytomas are some types of neoplastic diseases
observed in these test animals.

Additionally, the cost of maintaining animals during the long latency
period required for manifestation of the carcinogenic event to occur, is
considerable. Thus, the evidence from animal assay systems suggest that
long-term exposure of these fuel components may pose a potential carcino-
genic threat to man.

In this segment of the contract, we proposed to evaluate the molecular
interactions of HZ, MMH, SDMH, UDMH, ANA, PANA, PBNA and BP with oncogenic
viruses in human cells and to correlate this effect with in vitro chemical
transformation proposed in Segment I. We have used Snyder-Theilen Feline
Sarcoma Virus (ST-FeSV), an oncogenic RNA virus which transforms human
cells in vitro in measurable and predictable dose response kinetics (5).

The importance of this study may be the development of an in vitro
assay system with human cells that can quantitatively screen potential
co-carcinogens within a short-period of time (9-13 days). All chemicals
not having a carcinogen capability may indeed be co-carcinogens. This
procedure should identify those components in jet and rocket fuel that
are co-carcinogens.

We previously showed that combinations of hormones, carcinogens and an oncogenic DNA virus, SV40, enhanced virus transformation depending upon dose and time of application (6,7). We concluded from these studies that at least certain chemical carcinogens enhanced viral transformation by increasing the number of sites for integration of viral genetic information into cellular DNA (6).

These observations suggest that at least certain chemical carcinogens enhance viral transformation by increasing the number of sites for integration of viral genetic material into cell DNA.

The above observations with a non-replicating virus system (oncogenic DNA virus) have not been as clearly substantiated with replicating oncogenic RNA virus systems. Nonetheless, Freeman and Price et al. (8,9) have independently demonstrated that murine leukemia virus infected rat and mouse cells undergo transformation following addition of carcinogens such as 3-methylcholanthrene (MCA), BP, and diethylnitrosamine (DENA). The implications of their studies are that the carcinogens activate the viral coded oncogenic information which may be inherent in the cells but require the helper functions of the leukemia virus. However, others (8,9) found that treatment of rat embryo cultures with MCA one to three weeks prior to addition of Rauscher leukemia virus (RLV) did not lead to transformation of these cells, whereas MCA treatment up to three weeks after RLV infection yielded transformation within 7 to 10 passages. These data indicate that the changes induced in the cell by certain chemical carcinogens were of a transient nature, and that the chemical treatment apparently did not permanently activate some endogenous agent which later participated in the process of virus transformation. The possibility of transient viral gene activation (derepression followed by repression) cannot be excluded.

One advantage of the RNA tumor virus system is that the temporal relationship that exists between addition of virus and chemical in the oncogenic DNA virus system is greatly diminished. Because the virus continues to replicate, chemicals may be added simultaneously, shortly after or at some later time to the ST-FeSV virus infected cell.

The evidence that many carcinogens enhance oncogenic DNA viral transformation in vitro suggested similar approaches be undertaken with human cells and HZ, MMH, UDMH, SDMH, ANA, PANA and PBNA. In this laboratory, the chemicals and carcinogens demonstrated to enhance viral transformation of hamster, mouse and rat cells were examined for similar effects with SV40 virus (7) and were examined with ST-FeSV (10). Our studies to date have revealed a good correlation between the ability of an in vivo carcinogen to enhance oncogenic DNA virus transformation chemicals included in this group, include BAP, MNNG, 4NQO, Acetoxy AAF, but not MCA nor 7,12 DMBA (7). However, the results found with the ST-FeSV human cell transforming system demonstrated a marked inhibition of virus directed transformation (10-12).

Endogenous C-type RNA viruses have been observed by electron microscopy in 18 different mammalian species including humans, several avian species, snakes and fish (13). Their presence as integrated genomes in vertebrates suggested these viruses served as regulators of natural life processes (14).

Endogenous C-type virus release has been shown by Panem et al. (15), to occur sporadically in normal human fibroblasts and evidence has been provided by this group, that endogenous viruses may play a role in at least one human autoimmune disease. Further, the expression of endogenous virus genes appear to be under cellular control. We have recently shown using

6

FeSV transformation of human cells, that select chemical carcinogens may interfere with host cell translational mechanisms, proviral synthesis or integration (16).

Since carcinogens may interfere with these functions, they may also interfere with gene products that function as suppressors of endogenous virus expression. Thus, normal cells treated with chemical carcinogens may induce expression of endogenous viruses whose presence may be detectable by electron microscopy, simultaneous detection of 70S viral RNA and RNA directed DNA polymerase, or immunological techniques.

In murine cells, endogenous viruses induced by halogenated pyrimidines or protein inhibitors, may be detectable from 12 hours to 96 hours post treatment. Thus, if endogenous virus release is detectable after chemical treatment and correlated with carcinogenic potential of the chemical, a rapid sensitive assay may be developed to screen potential carcinogens.

Steroid hormones have been reported to inhibit viral infection in vitro (19), enhance or inhibit viral oncogenesis in animal species (20), enhance or inhibit viral transformation in vitro (6,22), modulate oncogenic RNA virus expression in non-permissive cells (23) and to inhibit DNA repair (excision) with increased virus transformation in estrogen and chemical carcinogen treated SV40 virus infected human cells (6).

The mechanisms of hormonal action suggest the hormone enters the cell by diffusion, where it binds with low molecular weight proteins or receptor molecules present in cell cytoplasm. The hormone-receptor complex with increased affinity for chromosomal sites is translocated to the nucleus, whereby an undetermined mechanism, gene expression, is modulated.

Since hormones modulate gene expression and have been shown to affect oncogenic expression of both chemical transformation (Milo Segment I, this report) and virus transformation (6), their interaction with known carcinogens which may interfere with host cell translational mechanisms may result in endogenous viral expression in human cells.

IV. Rationale

Excellent reviews on the molecular biology of transformation of mammalian cells by oncogenic RNA and DNA viruses have been written and will not be discussed at length here (17-19).

Almost without exception, malignant transformation requires that at least a segment of the infecting viral genome, in the case of RNA viruses, the DNA provirus, becomes integrated into the host cell genome. Integration requires an alteration in at least the primary structure of cellular DNA. For transformation to occur, cellular DNA synthesis following viral integration is required (18).

A major difference in cellular transformation by oncogenic viruses and chemical carcinogens is that new genetic information is added to the cell by the former and existing genetic information is modified by the latter. However, the modification of genetic material by chemical carcinogens may also activate (derepress) pre-existing endogenous oncogenic viral genes.

A. Feline Sarcoma Virus. The Snyder-Theilen strain of Feline Sarcoma Virus produces progressively growing sarcomas in cats, dogs, sheep, rabbits, pigs, rats and monkeys (23-29). Cells from these species as well as from oxen (30) and humans (31-34) are transformed in vitro.

FeSV is an oncornavirus, and like other such viruses, stimulates cellular DNA synthesis during transformation of infected cells. Although it has not been shown directly that FeSV like other oncornaviruses forms a DNA pro-viral replicate that becomes integrated in the cell genome, it seems a reasonable assumption that this also occurs with FeSV (35). Indirect evidence supporting this assumption is the formation of sarcoma positive, leukemia negative (S+L-) mouse cells transformed by Moloney murine sarcoma virus (MMSV) which produces lytic foci in reaction to infection by ecotropic MuLV (36). A similar S+L- cell line has been produced by infection of cat cells with MMSV and is insensitive to both replicating MuLV (xenotropic) and feline leukemia virus (37).

Criteria commonly used for determining genetic expression in transformed cells (endogenous and exogenous) C-type RNA viruses, has been the serologic detection of specific (38) or interspecies specific antigens (39). With regard to transformation by C-type RNA viruses, the expression of tumor-specific antigen (FOCMA) (39), abnormal colonial morphology or foci of transformed cells as expressed by loss of contact inhibition and/or growth in soft agar, have been used as a means to judge the transformation event (39,40).

This laboratory has demonstrated the reproducibility of _in vitro_ transformation (focus formation) by Snyder-Theilen Feline Sarcoma Virus (ST-FeSV) in Detroit 550 human skin fibroblasts. Transformed foci appeared as rounded hyper-refractile cells and were observed to follow the pattern of monolayer cell growth. Giant cell formation was noted 14 days p.i.

Reproducibility of this cell-virus system permitted us to study the role chemical carcinogens and oncogenic RNA viruses play in transformation.

Because of our extensive experience with this virus _in vitro_ and
in vivo, we choose to proceed with it in lieu of other RNA sarcoma
viruses. This approach is predicated on the basis that the mechanisms
involved in promotion of RNA tumor virus transformation by chemicals
or vice versa should be nearly identical with all RNA tumor viruses.

The use of an RNA tumor virus in co-carcinogenesis studies is
justified on the basis that the process of integration of viral genetic
information, in order for transformation to occur, is more complex with
an RNA tumor virus. The requirement for synthesis of a DNA copy or
copies of the RNA genome will certainly alter time sequences between
addition of carcinogen and virus as previously discussed.

B. _Activation of C-Type Viruses by Chemical Carcinogens_. The evidence
for C-type RNA virus activation in mammalian and avian cells by various
means warranted analyses for endogenous RNA C-type viral expression in
human cells transformed by SV40 virus or treated with hydrazine and
derivatives and polycyclic hydrocarbons. A number of investigators have
reported induction of virus synthesis in normal cells by chemical, physical,
and viral carcinogens. Leukemia virus group-specific antigen (HaLV gs)
in hamster cell lines transformed by MCA or by certain fractions of
cigarette smoke condensates has been demonstrated. The transformed cell
lines were negative for infectious virus before inoculation into hamsters.
However, hamster-specific C-type RNA virus was isolated from tumors or
from cell lines derived from the tumors. Since C-type viruses were nor-
mally not found in either hamster tissue or hamster tumors, they concluded
the chemical treatment activated the virus.

Rowe _et al_. (41) reported the establishment of a virus-negative AKR
mouse embryo cell line which could be induced to yield virus by X-irradiation,

ultraviolet irradiation, or after transformation, by SV40 virus. Their findings suggest AKR cells carry the full RNA tumor virus genome in an unexpressed form.

Weiss et al. (42) induced avian tumor virus formation in normal chicken cells after treatment with ionizing radiation, chemical carcinogens and mutagens.

Spontaneous and chemical activation of C-type RNA viruses has been reported in cloned mouse cells (42,43), rat (44,45), cat (47-49), human tumor and normal human fibroblasts (50-52).

In certain instances, the virus isolates were oncogenic (53) and provided "helper" function in defective virus studies and possessed physical properties of the C-type RNA viruses (52).

Based on the above evidence of activation of endogenous C-type RNA viruses and viral gene expression in mammalian and avian cells, we determined whether treatment of normal human cells by hydrazine compounds and polycyclic hydrocarbons results in partial or complete expression of an endogenous human RNA virus. Detection of RNA dependent DNA polymerase activity (Reverse Transcriptase) and transmission electron microscopy (TEM) of fixed cell pellets were used to monitor expression

Cell Line Used in this Study. The rationale for using the Detroit 550 (D550) (male, human foreskin fibroblast) cell strain (American Type Culture Collection CCL 109) was described in FY 77 Annual Report, based on our previous work with this cell line in SV40 studies.

HZ, MMH, UDMH, SDMH, ANA, PANA, PBNA and BP were assayed as co-carcinogens in the ST-FeSV-D550 cell virus system to determine if the interaction of these compounds affect virus transformation in a predictable manner.

V. Scientific Progress on Milestones Attained in FY 79

1. Tested ANA, PANA, PBNA, HZ, MMH, UDMH and SDMH co-carcinogenesis assay system.

2. Determined dose and time relationships for napthylamines and hydra-zines in co-carcinogenesis assay.

3. Showed correlative effect of napthylamines, hydrazine and its derivatives with _in vitro_ chemical transformation, neoplastic transformation and inhibition of virus transformation.

4. Endogenous virus activation did not occur in short-term (60-day) exposure of normal cells to HZ, MMH, UDMH and SDMH.

VI. Final Report Summary (Segment II)

A. Results to Date (Segment II) on Virus Induced Neoplastic Trans-formation

1. Co-carcinogenesis studies

<u>Cells, Chemical Preparation and Co-carcinogenesis Assays</u>. Human foreskin fibroblast cells (Detroit 550-CCL109, American Type Culture Collection, Rockville, Md.) were grown in EMEM (Gibco, Grand Island, N.Y.) supplemented with 1 Mm sodium pyruvate, 1X non-essential amino acids, 2 mM glutamine, 50 µg gentamycin per ml (Schering Diagnostics, Port Reading, N.J.), 0.11% sodium bicarbonate, and 10% FBS (Sterile Systems, Ogden, Utah). Cells were serially passaged every 3 to 4 days at a 1:2 ratio and incubated at $37^\circ C$ in 5% CO_2 atmosphere.

Just prior to use, the ANA, PANA or PBNA were weighed and dissolved in spectral grade acetone at 10 mg/ml and a stock solution made by adding 1 ml to 100 ml pre-warmed ($37^\circ C$) E-MEM + 10 FBS at pH 7.0. HZ MMH or UDMH were pipetted directly into E-MEM + FBS, pH 7.0 at the desired working concentration (PPM). SDMH was dissolved in 0.1 N HCL

at 10 mg/ml and diluted in E-MEM + FBS to experimental concentrations.
Stock cell cultures were trypsinized and seeded into 35 mm wells at a
concentration of I X 10^5 cells/well in 4.0 ml E-MEM + 10 FBS and incubated
18 hours prior to treatment. Cells pre-treated with chemicals prior to
virus infection were incubated with designated concentrations of chemical
for 90 minutes at the following time periods: 24 hrs, 6 hrs and 2 hrs,
washed and treated with 1.0 ml of DEAE-dextran (40 μg/ml) in serum-free
E-MEM. After 20 minutes, the cells were rinsed with E-MEM + 5% FBS, in-
fected with 0.2 ml per well with each of four 2-fold virus dilutions and
allowed to adsorb for 2 hours. Four wells were used per dilution of
virus. The plates were rocked at 10 to 15 minute intervals to maintain
an even distribution of inoculum and after adsorption, the inoculum was
removed and replaced with 4 ml of growth medium. Cells post-treated with
chemicals after virus infection were incubated with designated concentra-
tions of chemicals 2, 6 or 24 hours for 90 minutes after virus adsorption.
The medium was removed, washed and refed with growth medium. Cells were
refed with fresh growth medium only on the 6th day after infection and
subsequently fixed with buffered formalin and stained with Giemsa 3 to 4
days later. Foci appear as discrete areas consisting of round, hyper-
refractile, enlarged fibroblast cells (10). These foci were counted at
25 to 40 X with a dissecting microscope.

In other experiments, the concentrations of chemicals used in these
experiments were determined by treating non-virus infected cells for 90'
to discern the appropriate concentration to use in the virus experiments.
Cells were washed, refed and incubated 9-10 days, fixed in buffered
formalin, stained with Giemsa and clones containing at least 50 cells
were counted. Toxicity (surviving fraction) was determined by dividing

the average number of clones in treated wells by the average number of
clones in control wells.

Virus-induced foci were counted in nontreated and chemically treated
wells. FFU $\pm$ S.D. were determined for each treatment time and signifi-
cance determined by Student's test. The figures in the text where percent
inhibition is presented was determined by: $100 - \dfrac{\text{FFU chemically treated}}{\text{FFU control}}$

(a) Time and Dose Related Effects of ANA on ST-FeSV Transformation

Virus-directed transformation was significantly inhibited by chemical
treatment (10 μg and 0.01 μg/ml) when cells were treated from 6 hours to
2 hours before virus infection, but not when treated 24 hours before in-
fection. Furthermore, in virus infected cells treated with ANA, inhibition
of transformation occurred at 2 hours post-infection (p.i.) but not at 6
or 24 hours p.i. (Fig. 1).

(b) Time and Dose-Related Effects of PANA on ST-FeSV Transformation

Three concentrations (20 μg/ml, 10 μg/ml, 0.1 μg/ml) inhibited
ST-FeSV transformation in human cells. Inhibition was dose dependent
and time-independent in that concentrations of 20 and 1.0 μg/ml signifi-
cantly inhibited transformation at all 6 time periods tested where the
lowest concentration (0.1 μg/ml) had no effect on virus directed trans-
formation at any of the 6 time periods used in this test (Fig. 2).

(c) Dose- and Time-Related Effects of PBNA on ST-FeSV Transformation

Four (4) concentrations of PBNA were assayed: (20, 10, 0.1 and
0.01 μg/ml). The highest concentrations (20 and 10 μg/ml) significantly
inhibited transformation at all 6 time periods with the greatest in-
hibitory effect seen when cells were treated before virus infection
(Fig. 3). Maximum inhibition resulted (71% and 60%) when cells were

exposed to 10 μg/ml, 6 hrs before infection and 20 μg/ml, 24 hours before infection, respectively. Lesser concentrations (0.1 and 0.01 μg/ml) enhanced transformation at 6 hours p.i.

Thus, at the 2 higher concentrations of PBNA the effect on virus transformation was inhibitory while at the 2 lower concentrations, the predominant effect was enhancement of transformation.

(d) MMH (100 ppm and 10 ppm) significantly enhanced virus transformation in a dose-dependent manner when cells were exposed 2 hrs pre-infection (Fig. 4). Conversely, treatment of virus-infected cells resulted in significant inhibition of transformation when cells were treated 2 hrs or 6 hrs p.i. No effect was noted at 24 hrs p.i.

(e) SDMH (100 μg/ml and 10 μg/ml) likewise enhanced virus transformation when cells were treated 2 hrs pre-infection (Fig. 5). In virus infected cells, SDMH treatment inhibited transformation by values ranging from 20% (10 μg/ml) to 30% (100 μg/ml). The inhibitory effect was observed at 2 hrs p.i., but not at 6 or 24 hrs p.i.

(f) The effects of UDMH on virus transformation are shown in Fig. 6. One hundred or 10 ppm exposure to UDMH inhibited transformation at 3 different time periods in relation to virus infection (Fig. 6). UDMH inhibited transformation by values ranging from 25% to 50% when cells were exposed 6 hrs before or 2 hrs and 24 hrs post-infection.

(g) The results of HZ (60 ppm and 6 ppm) exposure are shown in Fig. 7. Fig. 7 is a composite figure of 3 separate experiments showing HZ effects on virus transformation. Like SDMH and MMH, cells exposed to HZ 2 hrs pre-infection, significantly enhanced virus transformation whereas exposure 2 hrs post-virus infection significantly inhibited transforming effect of the sarcoma virus as did MMH, SDMH, and UDMH.

2. A. <u>Significance of Co-Carcinogenesis Studies in Relation to In Vitro Chemical Transformation and Neoplastic Transformation (from Segment I)</u>

Correlation of the above mentioned parameters with inhibition of virus transformation show that the test chemicals which chemically transform human cells <u>in vitro</u> which, in turn, produce neoplastic growth when injected in athymic mice, significantly inhibit virus transformation (Table 1). These results, when correlated with the data from Dr. Milo's work, suggest the napthylamines are carcinogenic when tested by the parameters listed in Segment I and II. HZ and UDMH showed a positive correlation in 3 of 3 listed parameters while MMH and SDMH did not (1 of 3). This data suggest ANA, PANA, PBNA, HZ and UDMH are carcinogens while SDMH and MMH have co-carcinogenic activity.

B. <u>Endogenous Virus Activation</u>

The results of attempts to activate endogenous human virus from HZ, MMH, SDMH and UDMH treated cells are shown in Table 2. Cells were constantly treated for 60 days and supernatants monitored for presence of RNA-directed DNA polymerase activity (Reverse Transcriptase) as a measure of virus expression. At the end of the experiment, the cells were removed from the flasks, processed for electron microscopic analysis, and examined for the evidence of "budding" C-type virus. The RDDP results indicated no virus was released and analysis of treated cells by TEM showed no virus release from treated or untreated cells (Table 3).

C. <u>Cytotoxic Analyses of Petroleum and Shale Oil Derived Fuels</u>

Petroleum derived (PD) or shale oil derived (SOD) JP5 or diesel fuel marine (DFM) fuels cytotoxic analyses are shown in Table 3. No significant difference in LD_{50}'s were detected for PD or SOD fuels. The LD_{50}

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for SOD-JP5 and PD-JP5 were 102 ppm and 100 ppm respectively while SOD-DFM and PD-DFM were 87 ppm and 85 ppm respectively. LD_{50} for JP10 fuel was 91 ppm while RJ5 was 19 ppm.

VII. Discussion

The co-carcinogenic effects of hydrazine and its derivatives and napthylamines described in this report, when correlated with in vitro chemical transformation and neoplastic transformation, show a high degree of correlation.

ANA, PANA, and PBNA showed 100% correlation with the 2 parameters, whereas MMH and SDMH showed activity in the co-carcinogenesis assays, but not in the carcinogenesis assays. HZ and UDMH, like the napthylamines, showed 100% correlation with in vitro chemical transformation and neoplastic transformation. The inhibition of transformation from chemical treatment was not a result of cell killing in that subtoxic concentrations were used.

Enhanced virus transformation by HZ, MMH, and SDMH observed when cells were exposed 2 hrs pre-infection, may be related to cell growth stimulation shown by these chemicals in dose survival studies (data not shown). Similar findings of cell stimulation have been observed with murine and feline lymphocyte cultures (54). The major effect on virus transformation (inhibition - anti-carcinogenic) occurred with all test chemicals when virus infected cells were exposed to the appropriate concentrations. The temporal relationship of chemical treatment to virus infection appears more critical with the hydrazines than with the napthylamines in that maximum inhibition occurred when virus infected cells were exposed to the hydrazines 2 hrs post-infection, whereas inhibition was observed at all 6 time periods with PANA and PBNA and at 3 time periods with ANA.

HZ, MMH, UDMH, SDMH and PANA have shown mutagenic, teratogenic or car-
cinogenic properties depending upon the assay used (55-59). Thus, these
chemicals interact with host cell transcriptional or translational pro-
cesses. In previous studies we concluded the inhibitory (anti-carcinogenic)
effect of benzo(a)pyrene, Aflatoxin B, or N-acetoxy-2 fluorenyl acetamide
on virus transformation was not due to decreased cellular proliferation or
virus synthesis (10,59). Further, the anti-carcinogenic effect was abro-
gated when cells were exposed >24 hours post-infection. The temporal
relationship between infection and treatment suggested chemical interference
with FeSV proviral synthesis or integration into host cell DNA.

Contrasting results have been reported on the interaction of chemical
carcinogens and oncogenic RNA viruses. For example, in vivo studies showed
either an anti-carcinogenic (60,61) co-carcinogenic (62,63) or no effect
(64,65) on transformation depending on the virus or chemical used in the
experiments, whereas in vitro studies with rat or mouse cells showed
synergism (66-68).

We previously reported anti-carcinogenic activity with 3 known car-
cinogens on FeSV transformation of human cells in vitro. A recent report
by Rhim and Arnstein (69) described anti-carcinogenic activity of an
oncogenic murine virus on chemical-induced transformation of canine cells.

Thus, the mechanism(s) of chemical, viral or co-chemical-viral trans-
formation remain unknown and further studies are warranted to evaluate
these interactions.

VIII. Methods of Procedure in Segment II

1. Viruses

Snyder-Theilen Feline Sarcoma Virus (ST-FeSV) - Feline sarcoma virus
containing tumor tissue was supplied by Dr. G. Theilen and has undergone

2-4 *in vivo* passages in our laboratory. Ten to 20% suspensions of minced tumor tissue in Leibowitz medium (L-15) were homogenized for 2 minutes and centrifuged at 2300 x g for 20 minutes. Supernatants were recentri- fuged as before, and the resulting supernatant centrifuged at 18,000 x g for 10 minutes. Final supernatants were passed through 0.45 μ filters or placed over sucrose for further purification. In the latter case, super- natants were layered over 5 ml of 45% sucrose (density = 1.2 g/ml) and centrifuged at 40,000 x g for 3 hours. Resulting virus-containing bands were dialysed to remove sucrose, aliquoted and frozen at -85°C. Virus was also produced from feline embryo-infected cells.

2. Cells

(a) Human foreskin fibroblast cells (normal male) designated Detroit 550 (American Type Culture Collection, Rockville, Md.) were grown in minimum essential medium Earle's Salts (MEM(E)), supplemented with 1X non-essential amino-acids, 1 mM sodium pyruvate, 2 mM glutamine, 50 μg/ml Gentamycin and 10% heat-activated fetal bovine serum. For routine passage, cells were grown to confluency in 120 cm^2 prescription bottles, dispersed with 0.1% trypsin prepared in incomplete MEM and subcultured on a 1:2 basis. Cells used in transformation assays were between passage levels 15 through 25 (Phase II).

Feline Embryo Cells (FE) - Feline embryo cell cultures prepared from 30 day fetuses were used from passage 3-10 for growth and virus quantita- tion assays with FeSV. Embryos were finely minced and trypsinized for 45 minutes. Dispersed cells were washed in L-15 medium containing 15% fetal calf serum and seeded into 400 ml prescription bottles. Early passage cultures were frozen at 102 x 10^6 cells/ml in 2 ml ampoules for constant supply of low passage cells.

3. Transformation Assays

a. Snyder-Theilen Feline Sarcoma Virus - Detroit 550 cells were seeded at concentrations of $1-2 \times 10^5$ cells per 35 mm wells in 4 ml of growth medium. After incubation for 24 hours, cells were pre-treated with 1 ml of DEAE-D dextran at 40 µg/ml for 20 minutes at room temperature, and washed with MEM(E) containing 5% FBS. Monolayers were infected with 0.2 ml of appropriate virus dilutions and allowed to adsorb for 2 hours. Inoculum were then removed and replaced with 4 ml of MEM + 10 FBS. In-fected cells were refed after 3-5 days. Foci of altered, hyper-refractile cells confined to discrete areas appear within 6-8 days. Foci were enumerated at 12-14 days by fixing with buffered formalin and staining with Giemsa.

4. Test Materials

Napthylamines were suspended in acetone (10 mg/ml), SDMH in 0.1 NHCL (10 mg/ml) and hydrazines in growth medium at 10^6 ppm and added to target cells at pre-determined dose levels. In previous studies in this laboratory with chemical carcinogens and mutagens, stock solutions of water-insoluble chemicals were dissolved in acetone at 10 mg/ml and a stock solution made by adding 1 ml to 100 ml prewarmed complete medium at pH 7.0 (10). Further dilutions were made in complete prewarmed medium to obtain the needed con-centration. This procedure was used in this study where properties of test materials permit. Subtoxic dose levels were determined by cell-survival studies with target cells by comparison of cloning efficiencies of treated vs. untreated cells.

5. Electron Microscopy

a. Thin section. Cells were removed from the glass either by trypsinization or scraping, washed in PBS and centrifuged at 150 x g for

10 minutes in an International centrifuge. Cell pellets were fixed with either 2% glutaraldehyde followed by 1% osmium tetraoxide or Dalton's chrome osmium fixative (70). After dehydration in graded ethyl alcohol, the cells were embedded in an Epon araldite mixture by the procedure of Mollenhauer (71) and thin sectioned with glass knives on a Porter-Blum microtome. Sections were stained with 2% uranyl acetate and with lead citrate and examined with a Phillips Model 200 or 300 electron microscope (72).

b. Negative staining techniques. Equal volumes of suspected virus suspensions were thoroughly mixed with 0.5% phosphotungsic acid solution (PTA) containing 0.2% sucrose, pH 7.0. Carbon coated colloidian covered grids were dried and scanned with the electron microscope at a magnification of 30,000 X.

6. Reverse Transcriptase Enzyme Assay

20-30 ml of chemically treated virus infected tissue culture media were clarified at 10,000 g for 15 min at $4^{\circ}C$. Clarified supernatants were then centrifuged at 100,000 g for 90 min at $4^{\circ}C$ to pelletize any virus present. Virus pellets were triturated in 50-80 μl of Tris-Triton suspension buffer (5 mM Tris-HCl at pH 8.1; 1 mM dithiothreitol (DTT); 0.1% Triton X-100 and 0.5 M KCl. 10 μl aliquots of the above virus suspension were incubated in a $37^{\circ}C$ water bath for 60 min in a final volume of 50 μl containing: 5 μl of Poly(rA)$_n$ (0.3 μg) premixed with 4 μl of Oligo(dT) 12-18 (0.4 μg) and 1 μl of H_2O; 5 μl of 50 mM Tris - 0.1% Triton X-100, 5 μl of 1 mM manganese acetate, 5 μl of 20 mM DTT, 5 μl of H_2O and 10 μl of (5 μCi) (^{3}H) thymidine triphosphate (TTP) (spec. act. 17 Ci/mmol). Following incubation, 40 μl of the reaction mixture were spotted on Whatman 3 filter paper disks, washed 4 times with 500 ml cold

acid wash (10% sodium pyrophosphate, 0.7% hydrochloric acid and 5% glacial acetic acid) and a final wash in 500 ml of a 1 : 1 mixture of ethanol-ether for 10 min. The disks were dried and (^{3}H) TTP incorporation measured by scintillation counting the cold acid precipitable material in 10 ml scintillation cocktail composed of 15.2 g BBOT in 1 gal toluene.

IX. Support Data

1. 16 copies - "Induction of Retrovirus Non-Producer Human Cells to Producer Cells by Dexamethasone". By: JR Blakeslee, A Elliot and D Turner

2. 16 copies - "Factors Affecting Feline Retrovirus Infectivity and Oncogenicity." In: Feline Leukemia, Ed. RG Olsen, CRC Press, Boca Raton, Fla. In Press, 1980

Table 1. Correlation between Inhibition of Virus Transformation, In Vitro
Chemical Transformation and Neoplastic Transformation

Chemical	In Vitro Chemical Transformation	Neoplastic Transformation	Inhibition of Virus Transformation
ANA	Yes	Yes	Yes
PANA	Yes	Yes	Yes
PBNA	Yes	Yes	Yes
BP*	Yes	Yes	Yes
Pyr.**	No	No	No
HZ	Yes	Yes	Yes
MMH	No	No	Yes
UDMH	Yes	Yes	Yes
SDMH	No	No	Yes

*BP - Benzo(a)pyrene

**Pyr. - Pyrene

Table 2. RDDP Activity in Cell Supernatants from HZ, MMH, SDMH and UDMH
Treated Cells

EXPERIMENT 1

Day Post-Treatment	(Untreated)	HZ (60 PPM)	MMH (80 PPM)	UDMH (100 PPM)
			(CPM x 10^{-3} of 3H)	
12	2.1	2.1	2.3	2.3
18	1.6	2.0	1.8	1.3
21	2.1	1.9	2.0	2.1
25	1.9	2.6	1.6	1.6
27	1.3	1.8	1.3	1.3
29	1.6	1.9	1.5	1.2
32	2.7	2.2	3.5	2.2
36	N.D.*	3.0	2.6	2.2
40	1.5	2.2	1.9	1.7
45	2.4	2.5	2.2	1.5
52	1.8	2.1	2.4	1.9
60	2.0	1.8	2.1	2.4

FeLV Standard $\overline{CPM}$ - 96.6

Background - $\overline{CPM}$ - 0.029

* - N.D. - Not Determined

EXPERIMENT 2

Days Post-Treatment	Untreated CPM x 10^{-3}	SDMH (100 µg/ml) CMP x 10^{-3} of 3H
5	1.5	1.8
10	1.8	1.4
14	2.3	2.1
21	2.5	2.6
30	1.7	1.9
35	2.8	2.4
42	2.0	2.2
49	1.4	1.8
52	1.6	1.4
59	1.5	1.2

FeLV Standard $\overline{CPM}$ - 50.3

Background - $\overline{CPM}$ - 0.025

Table 3.   SEM Results from Cells Exposed to HZ, MMH and UDMH

Chemical	# virus positive* # grids examined
Hydrazine	0/10
Monomethyl hydrazine	0/4
Unsymmetrical dimethylhydrazine	0/7
Symmetrical dimethylhydrazine	0/5

*As a positive control, F422 cells, a canine thymo-
cyte cell line which constantly sheds C-type feline
leukemia virus, were prepared in conjunction with
the chemically treated cells to control the fixation
procedure and to provide magnification and morpholog-
ical parameters.

Table 4. LD_{50} cytotoxicity of Shale Oil and Petroleum Derived Fuels in HSF Cells

Fuel	Derived From	LD_{50} (ppm)
JP5	Shale	102
JP5	Petroleum	100
DFM[1]	Shale	85
DFM	Petroleum	87
RJ5	Petroleum	19
JP10	Petroleum	91

[1] DFM = diesel fuel, marine

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Fig. 1. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated with ANA as described in Materials and Methods. (-) indicates cells treated before virus infection (hrs). (+) indicates cells treated after virus infection (hrs). * - significance determined by Student "t" test.

Figure 1
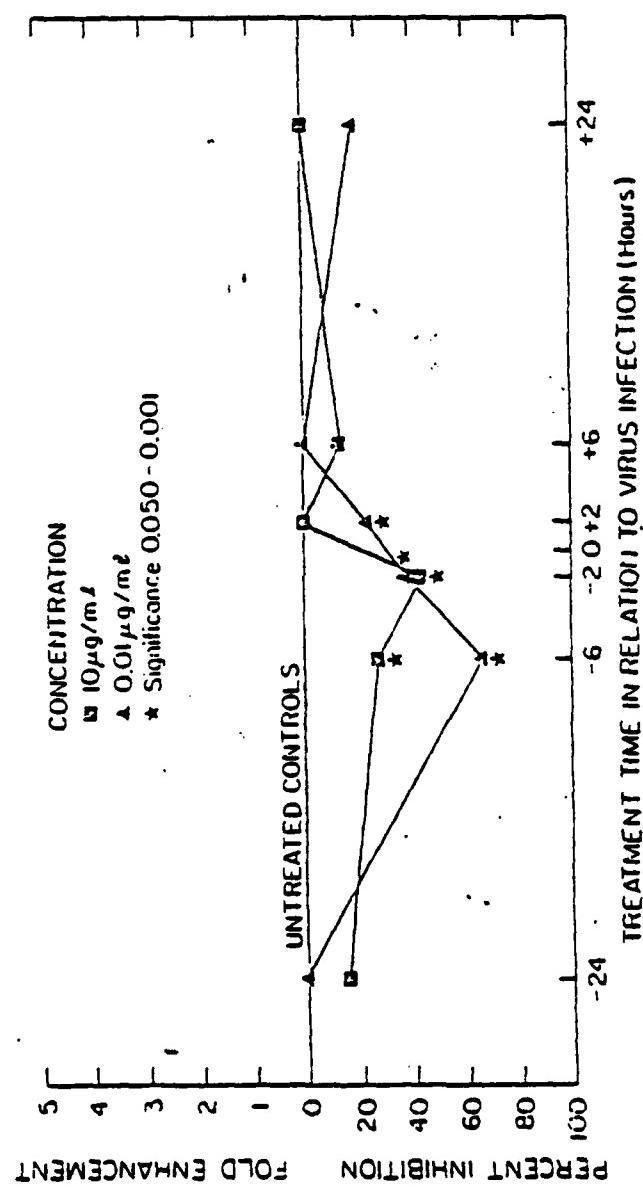
Fig. 2. Effect of PANA on ST-FeSV transformation. Cells treated with
PANA as described in Fig. 1.

Figure 2
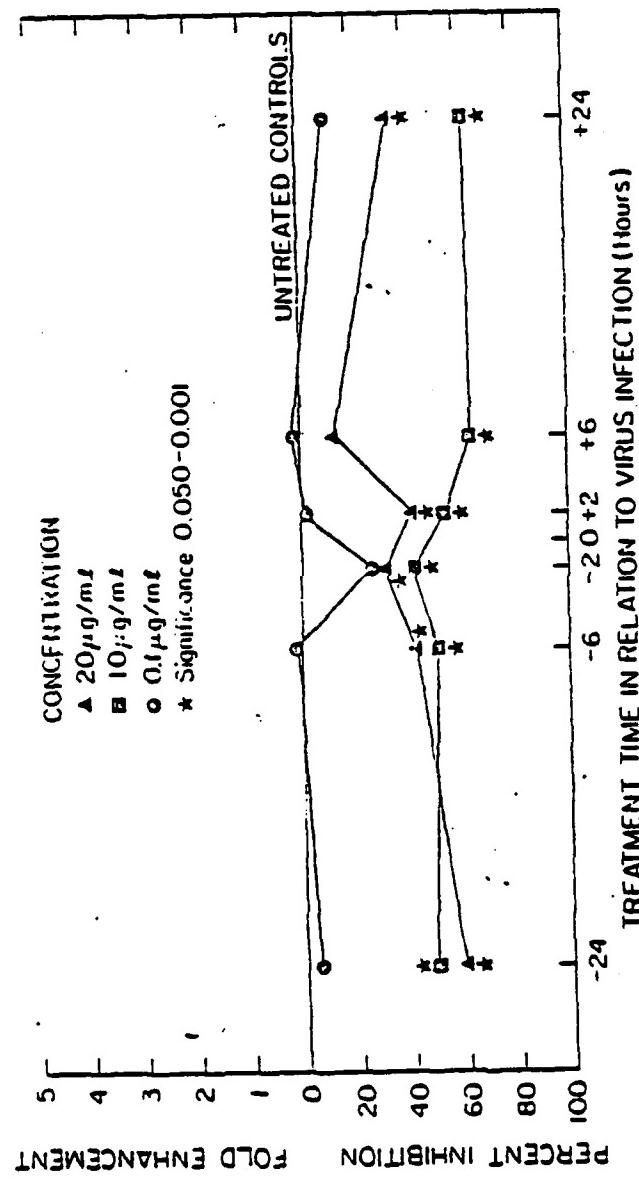
30

Fig. 3. Effect of PBNA on ST-FeSV transformation. Cells treated with
PBNA as described in Fig. 1.

Figure 3
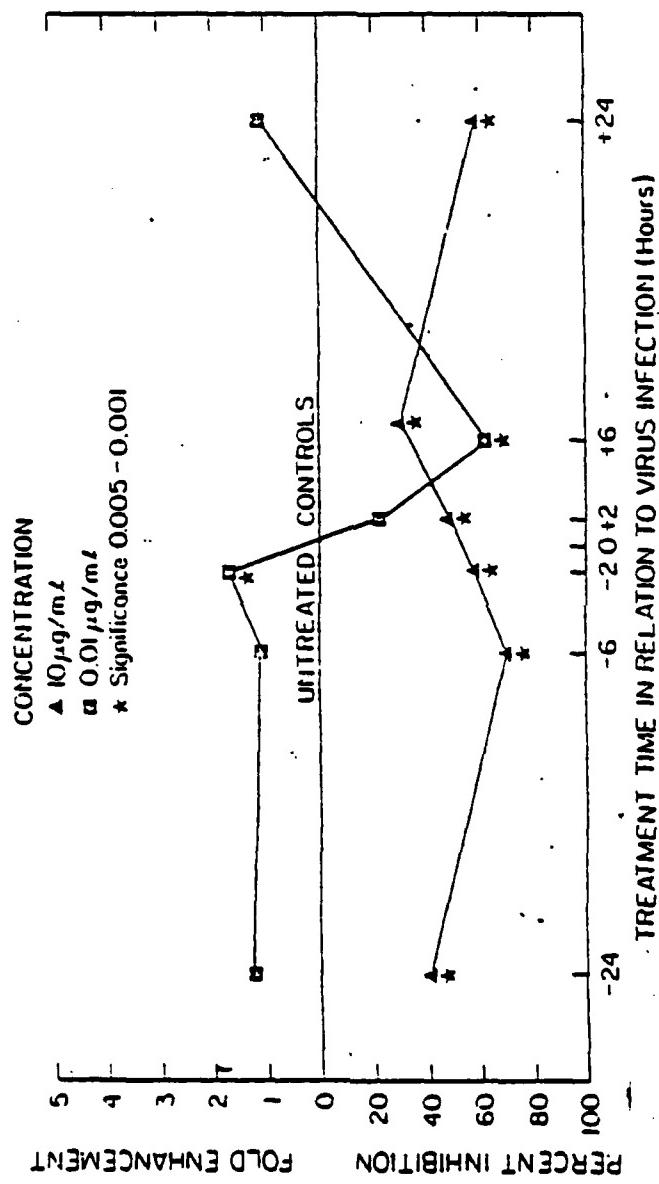
Fig. 4. Effect of MMH on ST-FeSV transformation. Cells treated with
MMH as described in Fig. 1.

Fig ^{33} 4
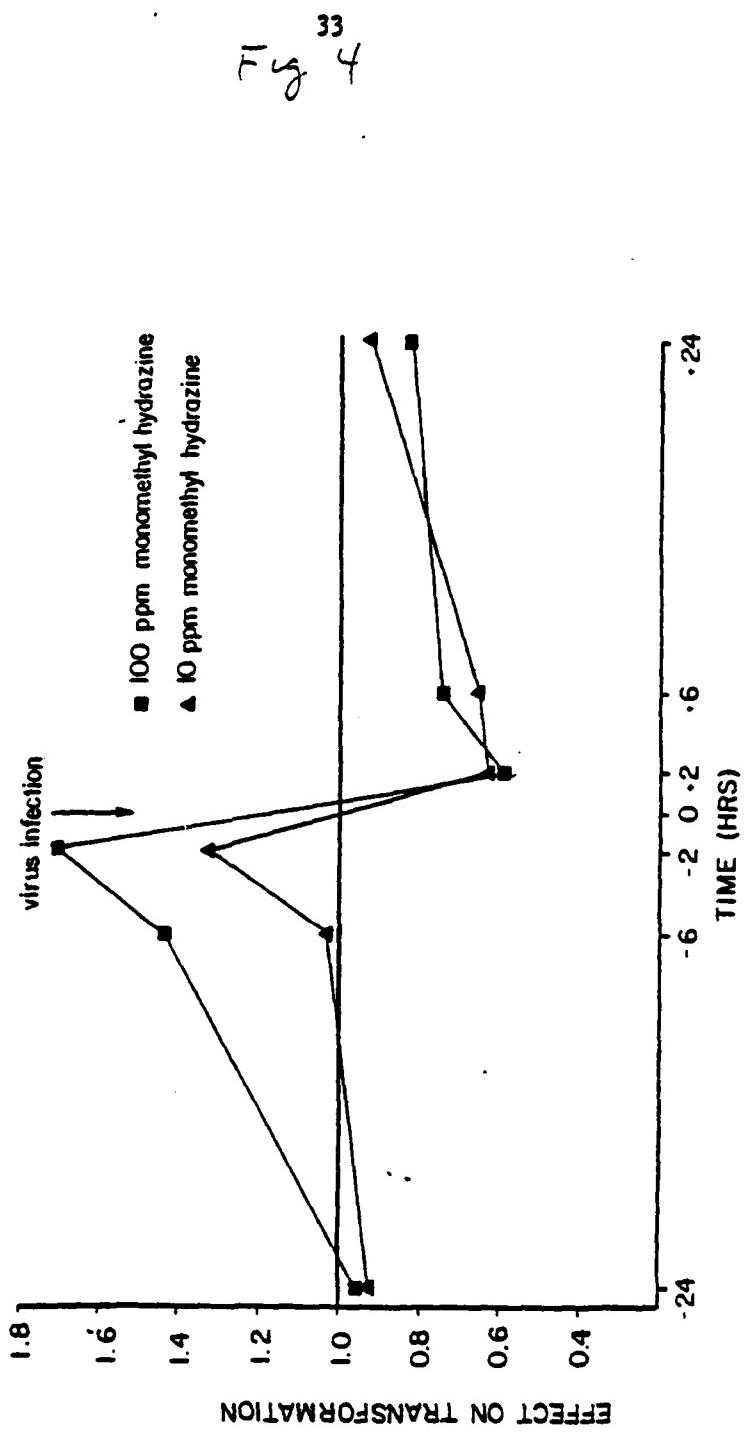
Fig. 5. Effect of SDMH on ST-FeSV transformation. Cells treated with
SDMH as described in Fig. 1.

EFFECT ON TRANSFORMATION

TIME (HRS)

■ 100 μg/ml sym. dimethylhydrazine
▲ 10 μg/ml sym dimethylylhydrazine
* significant

virus infection
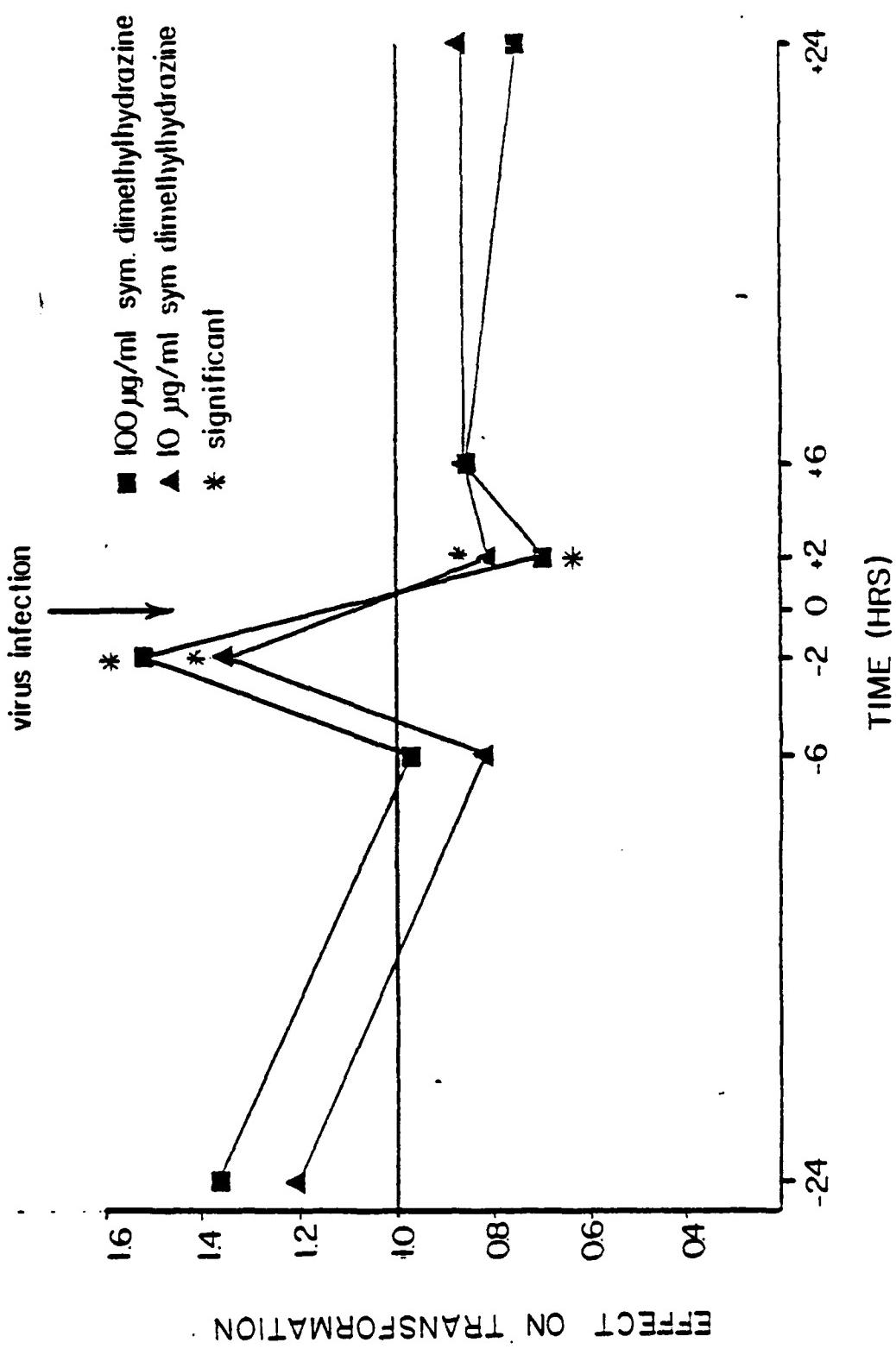
Fig. 6. Effect of UDMH on ST-FeSV transformation. Cells treated with UDMH as described in Fig. 1.

fig 6 37

Legend:
- ● 100 PPM - unsym. dimethylhydrazine
- ▲ 10 PPM - unsym. dimethylhydrazine
- * significant

Y-axis: EFFECT ON TRANSFORMATION (1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2)

X-axis: TREATMENT TIME (HOURS) (-24, -6, -2, 0, +2, +6, +24)

virus infection

** *
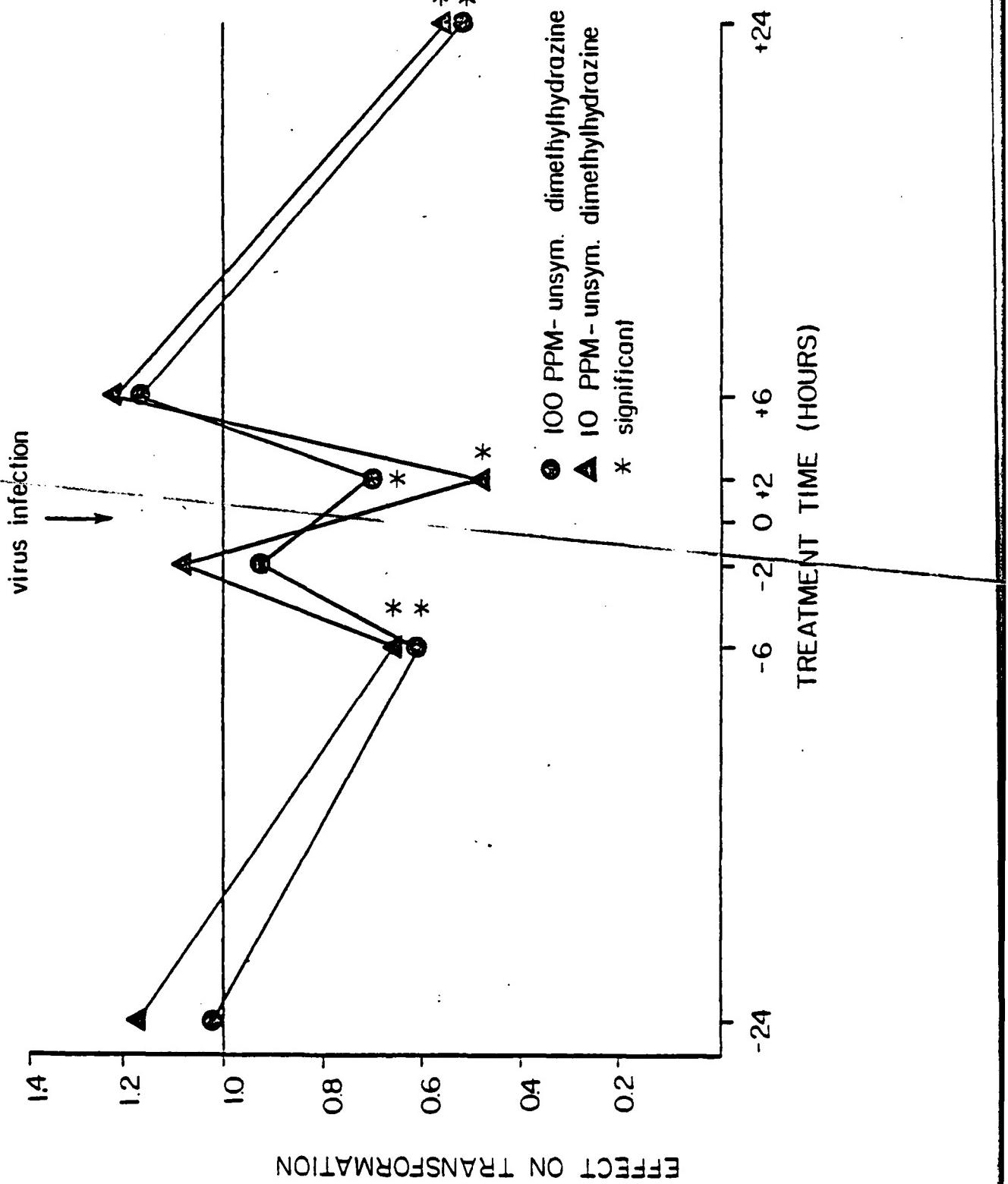
Fig. 7. *Effect of HZ on ST-FeSV transformation.* Cells treated with HZ as described in Fig. 1.

Fig 7 $\frac{39}{}$

virus infection

■ 60 ppm hydrazine
▲ 6 ppm hydrazine

TIME (HRS)

EFFECT ON TRANSFORMATION
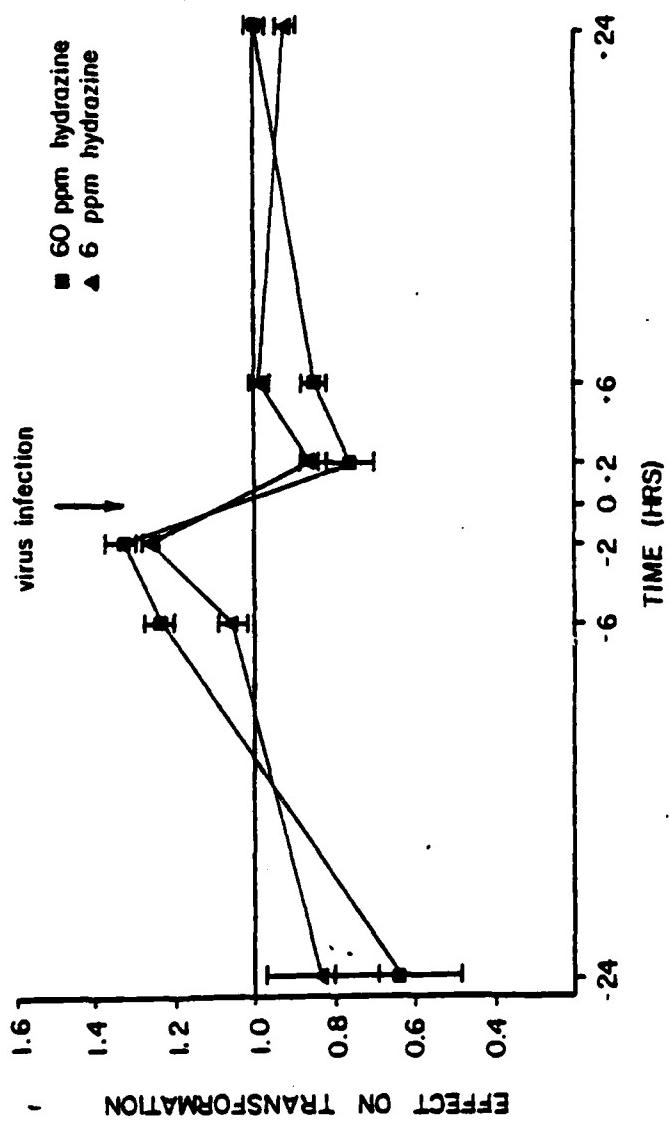
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Chapter 6

Factors Affecting Feline Retrovirus Infectivity and Oncogenicity

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Chapter 5.

Factors Affecting Feline Retrovirus Infectivity and Oncogenicity

Introduction

Adult cats which are normally resistant to challenge with the laboratory strain of feline leukemia virus (FeLV) can be made susceptible to FeLV infection and disease if exposed to certain chemicals. Similarly, productive infection or transformation by feline sarcoma virus (FeSV) or FeLV can be induced in normally resistant cultured cell lines by treatment with various chemicals or physical agents. The objectives of this chapter are to: 1) provide a brief background of chemical-viral cocarcinogenesis; 2) review the specific experiments involving the manipulation of FeLV-SV infectivity and oncogenicity by various means; and 3) discuss possible mechanisms of these changes in susceptibility to FeLV-SV in light of the reported experiments.

Background

Chemical alteration of viral infectivity or oncogenicity has been studied since the turn of the century. Many chemical carcinogens are known to permit or enhance viral-induced neoplasia or transformation both in vivo and in cell culture systems (1). For example, in mice, urethan in conjunction with murine leukemia virus (MuLV) caused a much higher incidence of leukemia (13-31%) than MuLV alone (0-2%), or urethan alone (4%) (2). Chieco-Bianchi et al. (3) showed similar synergistic effects with urethan and MuLV in their experiments.

Another example of in vivo chemical-viral cocarcinogenesis was demonstrated by Andrewes et al. (4) and Alhstrom and Andrewes (5), using rabbit fibroma virus and benzo-(a)-pyrene or tar. Application of the chemicals with the virus caused more numerous, larger and more slowly regressing fibromas than the virus or chemical alone.

Rous and Friedewald (6) showed similar results in rabbits treated with Shope papilloma virus followed by topical treatment with tar or 3-methylcholanthrene (3-MCA), resulting in the appearance of squamous cell carcinomas. These tumors did not develop after treatment with virus or chemical alone.

Similar cocarcinogenic effects have been found in certain cell culture lines treated with various physical or chemical agents and oncogenic viruses, particularly DNA viruses. Data presented by Casto and DiPaolo (1) using a Simian adenovirus and Blakeslee et al. (7) using SV40 virus showed that cells treated with virus and with ultraviolet light, various classes of chemical carcinogens, or DNA base analogues showed an increased susceptibility to viral transformation. It has been postulated that certain classes of carcinogens damage cellular DNA, and enhanced transformation is the result of viral DNA being present during host cell DNA repair synthesis (3). Alternatively, viral DNA may be incorporated into cellular DNA at sites of unrepaired lesions during scheduled DNA synthesis (9). The insertion of viral DNA in unrepaired sites could occur in cells defective in or with reduced repair capabilities.

These observations suggest that at least certain chemical carcinogens as well as radiation enhance viral transformation by increasing the number of sites for integration of viral genetic material into cell DNA.

The above observations with a nonreplicating DNA virus system have not been as clearly substantiated for replicative RNA tumor virus systems. Nonetheless, others (10) have demonstrated that murine leukemia virus infected rat and mouse cells undergo transformation following additon of carcinogens such as 3-MCA, benzo-(a)-pyrene (B(a)P) and diethylnitrosamine (DENA). These studies suggest that chemical carcinogens activate viral coded oncogenic information which may be inherent in the cells, but which require the helper functions of the leukemia virus for expression. However, the

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treatment of rat embryo cultures with 3MCA one to three weeks prior to additon of Rauscher Leukemia Virus (RLV) did not lead to transformation of these cells, whereas 3MCA treatment up to 3 weeks after RLV infection yielded transformed colonies within 7 to 10 passages. These data indicate that changes induced in the cell by certain chemical carcinogens were of a transient nature, and that the chemical treatment apparently did not permanently activate some endogenous agent which later participated in the process of virus transformation. The possibility of transient viral gene activation (derepression followed by repression) cannot be excluded.

In Vivo Enhancement of FeLV Oncogenicity by Methylnitrosourea: Occurrence and Possible Mechanism.

Adult specific-pathogen-free (SPF) cats are normally resistant to challenge with laboratory strains of FeLV, developing high feline oncornavirus-associated cell membrane antigen (FOCMA) and FeLV neutralizing antibodies, and transient or no viremia. Methylnitrosourea (MNU), a potent resorptive carcinogen of the nitrosamide family, was found to abolish this age-related resistance to FeLV when given in subcarcinogenic doses. In an initial experiment, 6 of 9 young adult cats treated with a single dose of 15 or 20 mg/kg MNU intravenously and inoculated with FeLV intraperitoneally (i.p.) became persistently viremic, while only 1 of 12 cats of the same age treated with FeLV alone became persistently viremic (11). Subsequent experiments showed that FOCMA antibody titers of FeLV + MNU treated cats were markedly lower than FeLV-treated cats (Table 1). It was further shown that the route of FeLV inoculation affected FeLV susceptibility in that the MNU-treated cats inoculated oronasally with FeLV showed a much lower incidence of viremia (1 of 5) compared to i.p. inoculation (6 of 9). However, MNU treatment of oronasally challenged cats did suppress FOCMA antibody titer (0.8 ± 0.5) compared to untreated oronasally challenged cats (4.0 ± 0.53).

A third experiment showed that MNU treatment did not alter pre-existing immunity to FeLV. Preimmune, MNU-treated cats were resistant to FeLV challenge, and developed a normal anamnestic antibody response to FOCMA. The results of all these experiments are summarized in Table 2.

Chemical carcinogens other than MNU have not been tested for their ability to enhance cats susceptibility to FeLV. Noncarcinogenic agents which alter FeLV susceptibility, such as corticosteroids and silica, are discussed in Chapter 4.

Immunosuppression Induced by MNU

One possible mechanism to explain chemical-viral cocarcinogenesis, in addition to those previously mentioned, includes chemically-induced general immunosuppression. An immunosuppressed host would be unable to respond adequately to viral antigens and/or viral-induced tumor antigens on transformed cells, hence, neoplasia could occur more readily. Many chemical carcinogens have been proven to be immunosuppressive (12) either because of systemic toxic effects or specific suppression of the reticulo-endothelial system.

The effects of MNU on the feline immune system were investigated and, indeed, MNU proved to be highly immunosuppressive (13). Cats were given a single dose of 15 mg/kg intravenously. Cutaneous allograft retention time was markedly prolonged, from an average of 16 days for the control group to an average of 74 days for the MNU-treated group (Fig. 1, Table 3). Lymphocyte blast transformation (LBT) response of peripheral mononuclear cells (PMC) to both antigen and mitogens was markedly suppressed for up to three months following MNU injection. The response to both pokeweed mitogen and concanavalin A was suppressed by up to 50-fold compared to the average pretreatment control values (Fig. 2 and Fig. 3). Similarly, the LBT response to specific antigen of cats treated with MNU and simultaneously immunized with keyhole
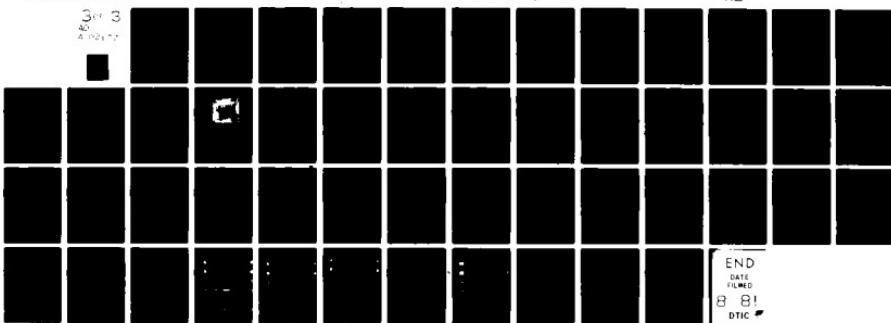
N acetoxy-2-fluorenyl acetamide (A-AAF) were investigated for their action on a quantitative virus directed focus forming assay (21).

As shown in Figs. 8 to 10, cells treated with sub-toxic doses of carcinogen resulted in significant inhibition of ST-FeSV directed transformation. Treatment with sub-toxic doses of the non-carcinogenic polycyclic hydrocarbon, pyrene, did not significantly reduce ST-FeSV focus formation, suggesting a possible relationship between carcinogenic potential and foci inhibition.

2. __Hormones.__ Steroid hormones have been reported to enhance or inhibit viral transformation _in vitro_ (7,20), modulate oncogenic DNA virus expression in nonpermissive cells (22) and to inhibit excision DNA repair with increased virus transformation in estrogen and chemical carcinogen-treated SV40 virus-infected human cells (7). Several reports have documented the stimulation of murine retrovirus synthesis in cell cultures exposed to optimal concentrations of glucogenic adrenocorticosterol hormones (23,24). More recently, Varnier and Levey (25) provided evidence that for xenotropic and the FMR strains of ecotropic endogenous murine leukemia viruses, replication is enhanced by dexamethasone (DXM). Schaller _et al._ (20) described quantitative and qualitative enhancement of ST-FeSV transformation in human neonatal foreskin cells when the DXM was added to infected cell cultures 24 hours post-infection. Additional hormones evaluated and shown to enhance focus formation were hydrocortisone, cortisol acetate and prednisone. No effect was detected with 17^β estradiol, progesterone, or methyl testosterone.

Blakeslee _et al._ (26) in further studies with DXM showed that non-DXM treated ST-FeSV infected human cells underwent morphological transformation (focus formation). However, little or no infectious virus was demonstrable in supernatant fluids from these cultures when added to the same strain of uninfected fibroblasts. The addition of 1.0 µg/ml of DXM 24 hours post-infection resulted in significant increases

(56X to 100X) in infectious FeSV (Table 4). Reverse transcriptase activity was likewise increased, ranging from a 9-fold increase to a 12-fold increase. Feline group-specific antigens (GSA) and FOCMA were detected in both DXM treated and nontreated FeSV infected cells. Uninfected cells, and cells treated with 0.2% acetone and/or DXM were negative (data not shown).

3. Asbestos. Asbestos is the commercial name for a group of naturally occurring, highly fibrous silicate minerals that readily separate into long, thin, strong fibers of sufficient flexibility to be woven. Industrial uses include cement, floor tiles, paper products, paint and caulking, brake linings, and cement-asbestos pipes.

Epidemiologic studies have shown occupational exposure can lead to increased risk of asbestosis, bronchogenic carcinoma, pleural mesothelioma and peritoneal mesothelioma. Ingestion or inhalation results in direct contact with epithelial cells lining the buccal cavity, esophagus, stomach and intestines.

As a continuation of studies in which FeSV transformation of human cells was used to develop an assay for determining potential carcinogens by their predictable effect on virus-induced transformation, 3 types of asbestos were used: Amosite, Chrysotile, and Crocidolite. Some properties are listed below:

Table 5. Characteristics of Asbestos Fibers.[1]

Characteristics	Chrysotile	Crocidolite	Amosite
Base Composition	Hydrate magnesium silicate	Hydrated silicate of iron and sodium	Hydrated silicate of iron and magnesium
Texture of fiber	Silky; soft	Harsh	Coarse
Flexibility and Spinning Properties	Excellent	Fair	Poor
Major Properties	Flexible heat resistant	Flexible heat resistant	Brittle

[1] Modified from Harrington, Allison and Badami

The results of experiments in which cells were infected with FeSV and treated either prior to or subsequent to infection with nontoxic concentrations of the 3 types of asbestos are shown in Figs. 11 to 13. Chrysotile- and Crocidolite-treated cells enhanced FeSV transformation (Figs. 11 and 12), whereas Amosite significantly inhibited transformation (Fig. 13).

As shown in Table 6, when virus was incubated with asbestos, then centrifuged to remove asbestos and the supernatant used to infect cells, a 60% reduction in virus transformation with Amosite was observed, suggesting adsorption of the virus to the Amosite. No such effect was noted with Chrysotile or Crocidolite. Concomitant treatment of cells with virus and asbestos resulted in significant enhancement of transformation (1.9 to 2.3 fold increase) with all 3 types (Table 7).

Relationship of Efficiency of DNA Repair, Age and Susceptibility to FeLV Infection

Various physical and chemical environmental agents have been shown to damage cellular DNA in vivo. Correctly repaired, the damage has little effect on the biological function of the system. Unrepaired damage, however, results in changes in physiological processes such as growth, transcription, mutation and induction of transformation. Thus, the more effective a cell is in the repair of genetic damage, the less sensitive it is to possible deleterious effects of environmental agents.

The efficiency of DNA repair can be measured by inducing DNA damage with ultraviolet light or chemicals, then measuring the rate of incorporation of tritiated DNA precursors into the repaired region. Using these techniques, less efficient DNA repair has been associated with the aging process and increased susceptibility to cancer.

Hart and Setlow (27) have related the expectant life (aging) of various mammalian species to the efficiency of DNA excision repair (one of three forms of DNA repair). In these studies, the initial rate of maximum incorporation of (^{3}H) dThd increased with life

span. Of the species tested, the amount of unscheduled DNA synthesis (DNA repair) was greatest in man, >elephant, >cow, >hamster, >rat, >mouse, >shrew. The extent of excision repair implied that cells proficient in such repair removed more damaged DNA than cells deficient in repair. Hence, over a given period of time, a mouse might accumulate in its DNA more damage per unit length than would a man, accounting for the differences in life span.

Increased susceptibility to virus-induced cellular transformation has been associated with the capacity for DNA repair. Blakeslee and Milo, in their studies with SV40 virus and chemical carcinogens, found that only those carcinogens which induced DNA damage enhanced SV40 transformation (28). Further, it was shown that hormonal inhibition of unscheduled DNA repair resulted in significant enhancement of virus transformation after DNA damage by a radiomimetic chemical (7).

Thus, the efficiency of DNA repair may have a direct relationship to the susceptibility of cells to viral integration and viral induced transformation.

Studies were undertaken to determine if a relationship existed between feline retrovirus induced disease and efficiency of DNA repair in feline fibroblast cells grown from surgical biopsies from different aged cats. Cellular DNA was damaged by one hundred ergs/mm^2 UV, scheduled DNA synthesis inhibited by arginine-free medium and hydroxyurea, and as a measurement of unscheduled DNA synthesis, ^{3}H-thymidine incorporation over a 24-hour time period was used to measure the extent and rate of repair.

The results of this study are shown in Fig. 14. The initial rate of repair up to 4 hours was similar regardless of the cat's age at the time of biopsy (Panels A & B). However, with increasing age, a decrease in the extent of repair was seen. Panel C depicts the results of repair in 3 littermates in order to determine variation within a

group of similar genetic make-up. As shown, no such variability was detected; the rate and extent of repair was similar with cells from the 3 kittens.

Discussion

Alteration of animal or cellular susceptibility to FeLV/SV infection by chemical or physical agents is a well recognized phenomenon. Several possible mechanisms are suggested by the preceeding experiments. Carcinogens, for example, may cause a generalized immunosuppression as shown in our experiments with MNU and cats, and this immunosuppression may account for the increased susceptibility to FeLV infection. In an immunocompetent animal exposed to FeLV, target cells may be continually infected and transformed, but the foreign viral-coded antigens such as FOCMA, which are expressed on the cell surface, elicit an immune response and the transformed cells are destroyed before they have a chance to become established. In an immunosuppressed animal, however, the transformed cells are less efficiently eliminated and thus able to establish a large population, resulting in neoplasia. To support this theory, most oncogens, when used in oncogenic doses, interfere with normal immunologic reactions (12).

On a cellular level, it appears that the cells involved in the immune response (lymphocytes and/or macrophages) are directly suppressed by carcinogens such as MNU, as evidenced by the decreased LBT response to con A after incubation with MNU. Other carcinogens which induce a dose-related suppression of the LBT response after or during in vitro incubation with lymphocytes include MNNG, hydrazine, and 1,1-dimethyl-hydrazine (Tarr, unpublished data).

Exposure to immunosuppressive compounds other than carcinogens, such as dexamethasone or silica, will also increase cats' susceptibility to feline retrovirus infection, as discussed in Chapter .

The use of in vitro cell culture systems allows the study of cell, virus, and chemical interactions at a molecular level, and may help elucidate the mechanisms of cocarcinogenesis. The experiments described in this chapter suggest several possible mechanisms.

Protein synthesis is one cellular function which may be affected by chemicals, resulting in an alteration of the normal host cell-virus relationship. Other investigators (29-31) have reported that carcinogens such as AFB1, A-AAF and B(a)P interfere with host cell translational mechanisms. Thus, chemical carcinogens may inhibit feline retrovirus-induced transformation by interfering with provirus synthesis or integration of proviral DNA into cellular DNA. In support of this concept, it is known that for avian and murine retroviruses, synthesis and transport of proviral DNA from the cytoplasm to the nucleus occurs between 6 and 24 hours post infection (32,33). An experiment described by Blakeslee and Milo (21) showed that B(a)P or AFB1 treatment 24 hours post-infection had little or no effect on transformation of FeSV-infected cells. This temporal relationship suggests that interference with proviral synthesis or integration has occurred.

Chemicals may also affect the normal interaction between virus and cell by altering viral gene expression. Wu et al. (34) described retrovirus infected cells as having 3 phenotypic categories with respect to virus gene expression: 1) virus producer cells, 2) non-producer cells with partial gene expression (any virus component) and 3) cells with no detectable viral gene products. Our experiments showed that BP or AFB2-treated and retrovirus-infected cells as well as cells infected with retrovirus alone produced no infectious virus, but demonstrated comparable levels of RT activity, and expressed FOCMA and GSA. These data indicate that: 1) the human skin fibroblasts used in these studies were classified as non-producer cells with partial viral gene

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expression, i.e. transformation, RT activity, GSA and FOCMA, 2) virus synthesis was not affected by the chemical carcinogen treatment, and 3) the inhibitory effect on virus transformation was chemically mediated while virus synthesis was host-cell mediated.

In contrast to B(a)P and AFB, DXM stimulated expression of all viral gene products, although not to the same degree. The RT activity was stimulated 9- to 12-fold, whereas release of infectious virus was stimulated 56- to 100-fold. This can be explained if there is partial gene expression (i.e. RT activity) without DXM treatment, which is indicated by the relatively high ^{3}H TMP incorporation in the RT assay using fluids from non-DXM treated, virus infected cells. Ahmed et al. (35) reported similar findings in that DXM-treated, Mason-Pfizer retrovirus-infected primate cells contained an 8-fold increase in RT activity and a 10-fold increase in infectious virus titer.

The increased virus synthesis in DXM treated cells is not the result of hormone-induced proliferations of cells in that previous studies by Schaller et al. (20) showed that 1.0 µg/ml DXM inhibited cell proliferation with increased yields of virus. The stimulatory-activity of DXM on retrovirus gene expression has been extensively studied and appears to be at a post-transcriptional step in virus replication (24,26,34,35,36).

The interaction of asbestos and feline retrovirus reveals yet another possible mechanism of chemical-viral alteration of cell activity. Electron micrographs show that all 3 fiber types are ingested by the fibroblast cells used in this study (37), and X-ray diffraction analyses of the treated cells revealed a loss of Mg^{++} and Fe^{+++} from the fibers within the cytoplasmic matrices. The order of loss was Amosite >Crocidolite >Chrysotile. Thus, leaching of the cations from the ingested asbestos fibers appears related to the effect on FeSV transformation in that cells treated with Amosite resulted in significant inhibition of transformation, whereas cells treated with Chrysotile and Crocidolite resulted in enhancement of transformation. The increased levels in Mg^{++}

may provide an increased cellular pool of cations required for viral replication and transformation (Crocidolite and Chrysotile) or, in the case of Amosite, a feed-back mechanism inhibiting cellular metabolic functions such as viropexis.

Intrinsic DNA repair mechanisms may also be a factor which affects cellular susceptibility to viral infection or transformation. On an organism level, there is a well established age-related susceptibility in cats to FeLV infection (38), with very young animals being most susceptible, and developing increasing resistance such that they are nearly refractory to challenge by 8-12 weeks of age. Our data using cells from different aged cats suggest that susceptibility to FeLV may be inversely related to excision DNA repair. During the leukemia susceptible period of up to 8 weeks of age, the extent of excision repair peaks at approximately 5 weeks, and by 10 weeks of age, a 4-fold decrease in the extent of repair was noted. Thus, during the period of susceptibility to FeLV infection, maximum repair synthesis also occurs. Further studies are required to determine if, during repair, more sites are available for FeLV proviral integration and whether increased FeLV genome equivalents are detectable in these cells.

The use of in vitro systems to study the mechanism(s) involved in the complex interactions between retroviruses and chemical and physical carcinogens in the induction of neoplastic disease may eventually lead to understanding the process of transformation of a normal cell to a neoplastic cell. However, certainly other factors must be considered, including genetic makeup, influence of age, environmental factors, physiological factors, stress, hormonal levels, and the host's immune response in the susceptibility of the cat to FeLV and FeSV infection and disease.

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Table 1.   Comparison of incidence of viremia and LSA disease and FOCMA antibody titer in cats inoculated intraperitoneally with FeLV and treated or not treated with MNU intravenously.

Cat No.	Rx	FeLV Viremia	Highest FOCMA of titer	LSA disease
597	FeLV	-	16	-
728		-	32	-
729		-	32	-
735		-	64	-
743		-	64	-
744		-	16	-
672	FeLV + MNU	+	4	+
676		+	4	+
701		+	4	+
703		-	64	-
706		+	4	+
707		+	4	+
718		+	4	+
734		-	128	-

Table 2. Proportion of Cats Developing Viremia, FOCMA Antibody, or LSA Disease Following Exposure to MNU and/or FeLV.

Treatment Groups	Mean Age at Exposure (mo)	No. of Cats Developing Persistent Viremia /Total No. Tested	No. of Cats Developing FOCMA Antibody /Total No. Tested	Geometric Mean Highest FOCMA Antibody Achieved	No. of Cats Developing LSA Disease /Total No. Tested
FeLV (IP)	6.2 ± 0.4	0/6 (0)[b]	6/6 (100)	20.0 ± 0.49[c]	0/6 (0)
FeLV (IP) + MNU	6.1 ± 1.0	6/8 (75)	1/8 (13)	1.3 ± 0.75	6/8 (75)
FeLV (O/N)	35.1 ± 20	0/8 (0)	6/8 (75)	4.0 ± 0.53	NA[d]
FeLV (O/N) + MNU	25.2 ± 15	1/5 (20)	1/5 (20)	0.8 ± 0.50	NA
FeLV (IP) + MNU (Pre-immune)[a]	7.5 ± 3.1	0/12 (0)	12/12 (100)	45.0 ± 0.51	NA

a) All 12 cats in this group had been exposed previously to FeLV at 4 mo. of age and carried persistently moderate to high FOCMA titers (mean = 24 ± 0.39).
b) Number in brackets represents the percent of cats responding.
c) Geometric mean FOCMA antibody titer ± standard error of the mean.
d) NA = not applicable.

Table 3. Effects of MNU on Cutaneous Allograft Retention.

MNU Treated Cats			Untreated Control Cats	
Animal No.	Graft Rejection Time		Animal No.	Graft Rejection Time
756	84d		747	15d
800	84d		813	17d
802	45d		814	17d
803	84d		819	16d
mean	74.25		mean	16.25d

20

Figure 1. Cutaneous allografts in MNU-treated and control cats.

A) Cat number 300B, 63 days after MNU administration and skin graft. Note white hair growth from edges of non-pigmented grafted skin.

B) Cat number 819B, control, showing complete graft rejection with necrosis and scaling 17 days following the grafting procedure.
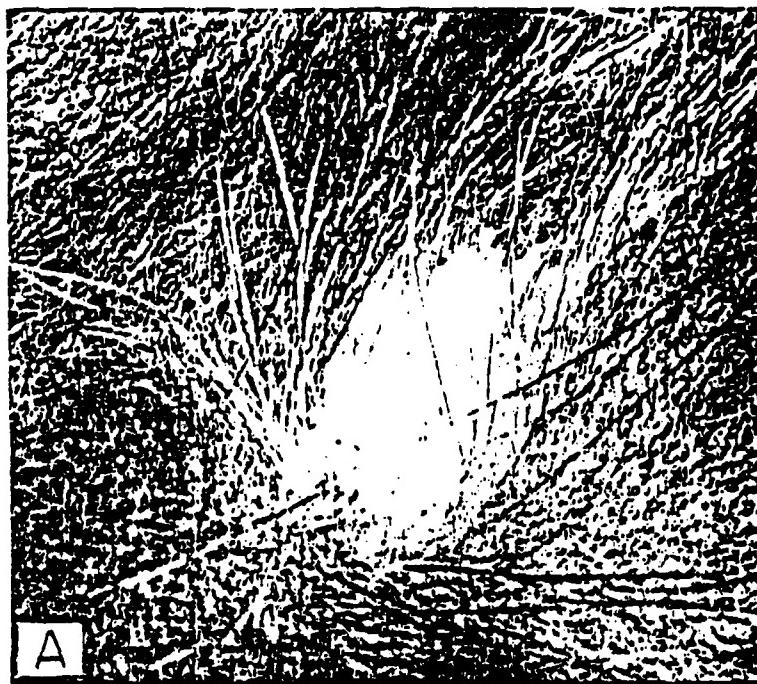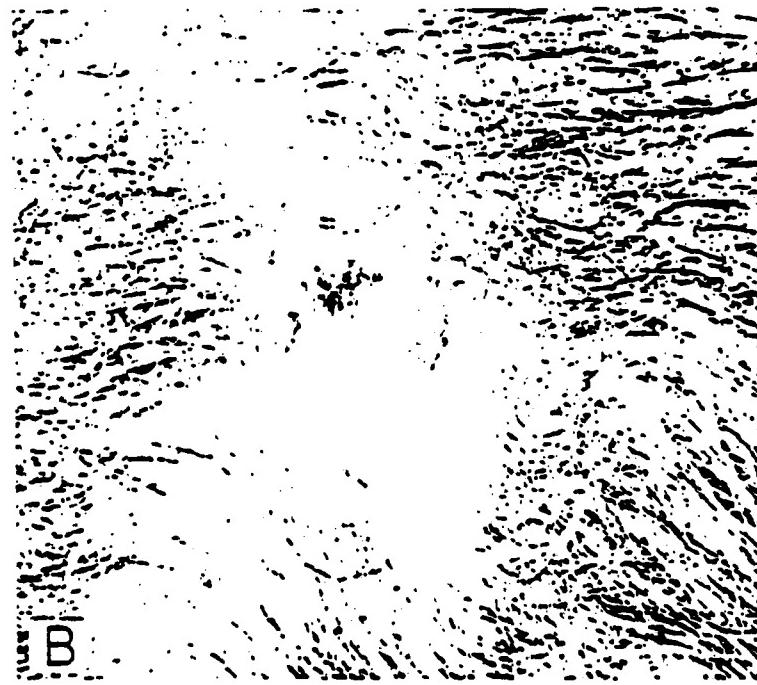
Figure 2. Lymphocyte blast transformation response of MNU-treated and control cats to con A. Vertical bars represent standard error of the mean (n = 4).

22
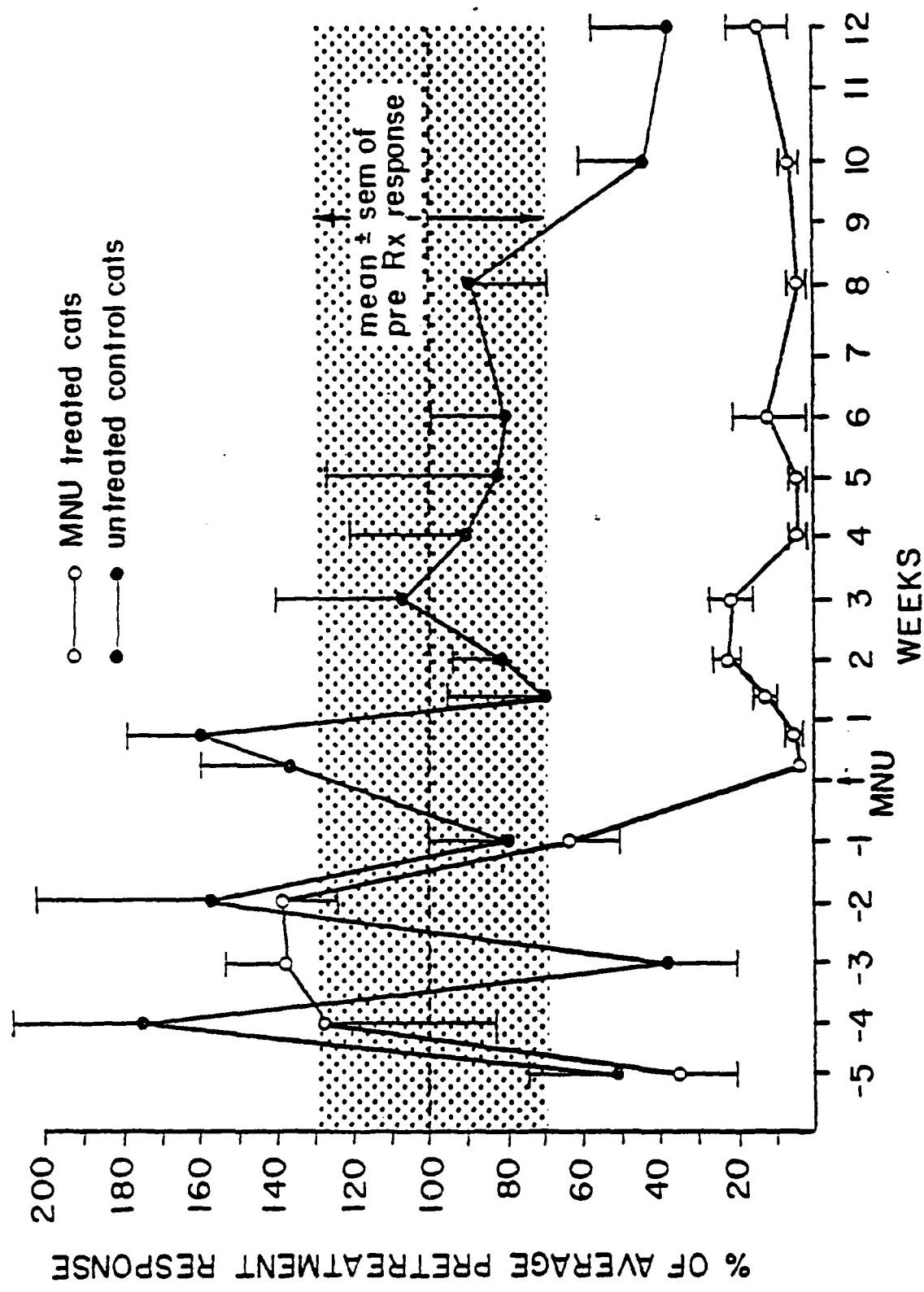
Figure 3. Lymphocyte blast transformation response of MNU-treated and control cats to PWM. Vertical bars represent standard error of the mean (n = 4). Rx = treatment.

% OF AVERAGE PRETREATMENT RESPONSE vs WEEKS

MNU treated cats (○)
untreated control cats (●)

mean ± sem of pre Rx response
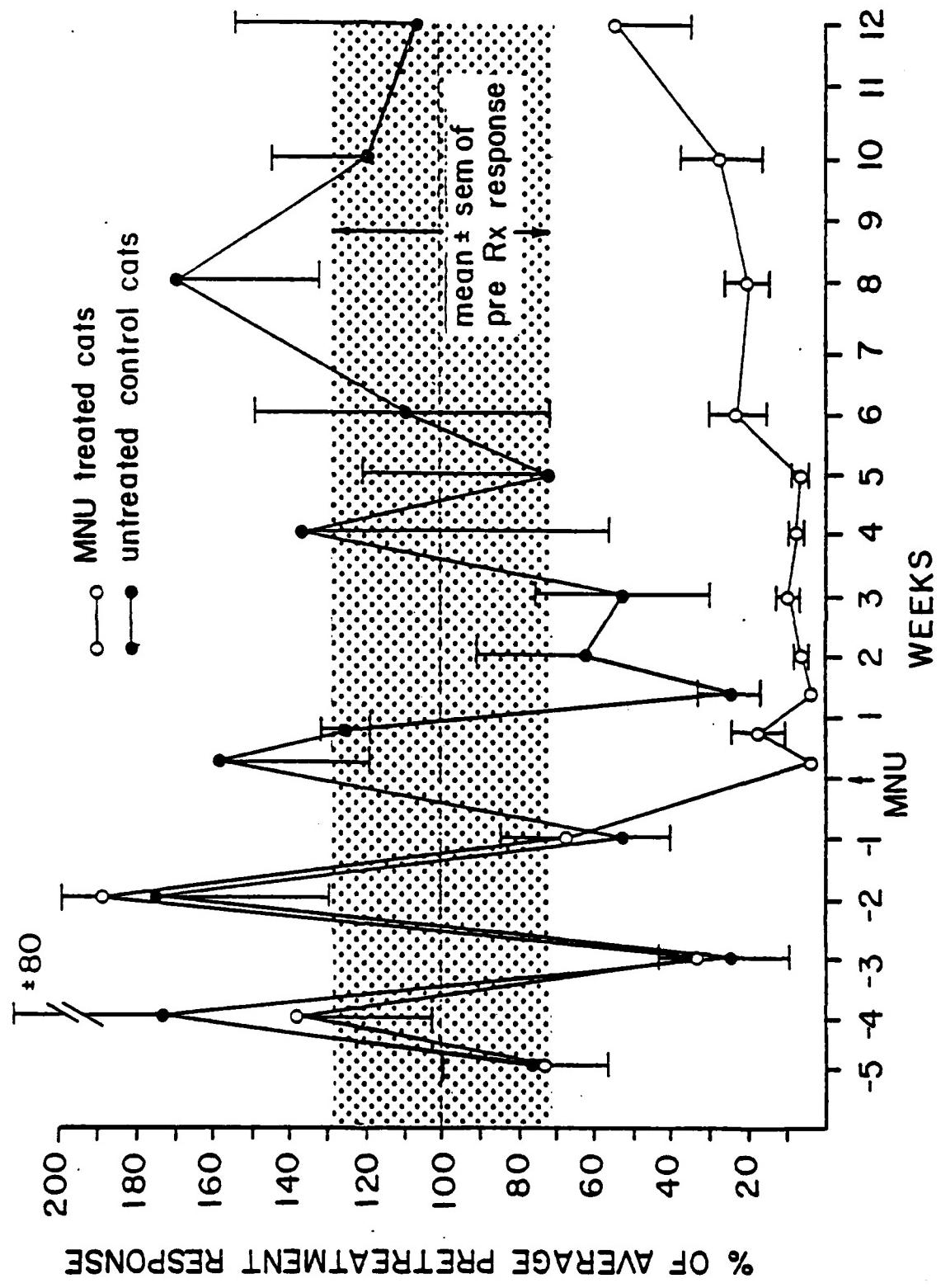
Figure 4. Lymphocyte blast transformation response of MNU-treated and control cats to KLH following KLH immunizaiton. Vertical bars represent standard error of the mean (n = 4).

24
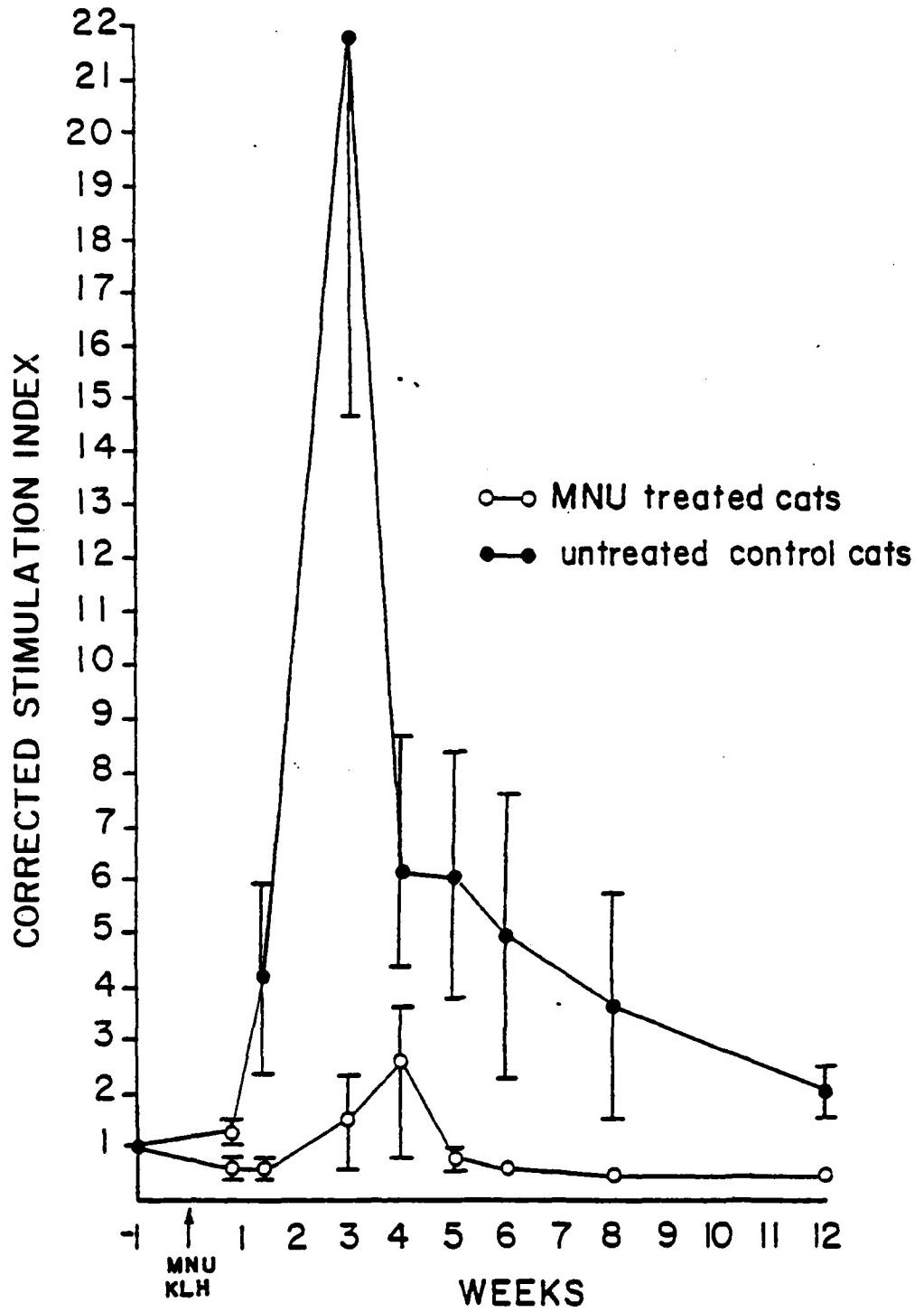
Figure 5. Effects of initial MNU incubation of PMC on LBT response to con A. Points represent (CPM of MNU + con A cultures/CPM of control con A cultures) X 100. Vertical bars represent standard error of the mean (n = 10).

PERCENT OF CONTROL RESPONSE

dose MNU μg/ml
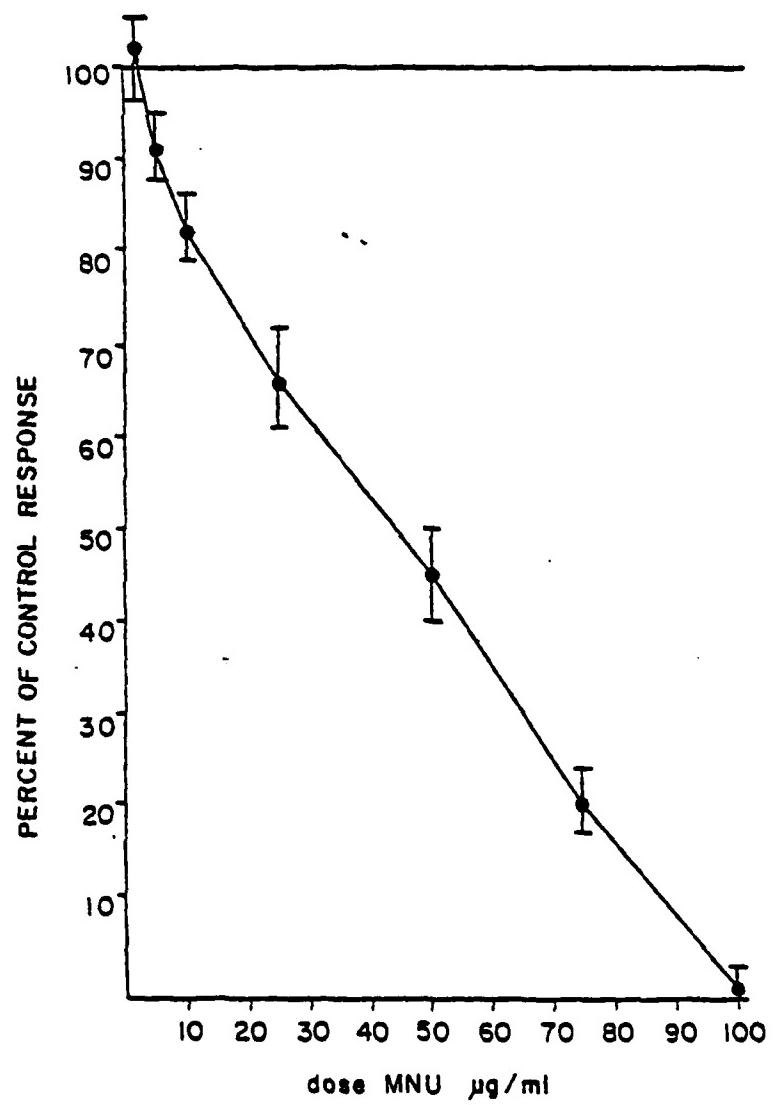
Figure 6. Effects of initial MNNG incubation of PMC on LBT response to con A. Points represent (CPM of MNNG + con A cultures/CPM of control con A cultures) X 100. Vertical bars represent standard error of the mean (n = 5).

PERCENT OF CONTROL RESPONSE

CONCENTRATION MNNG
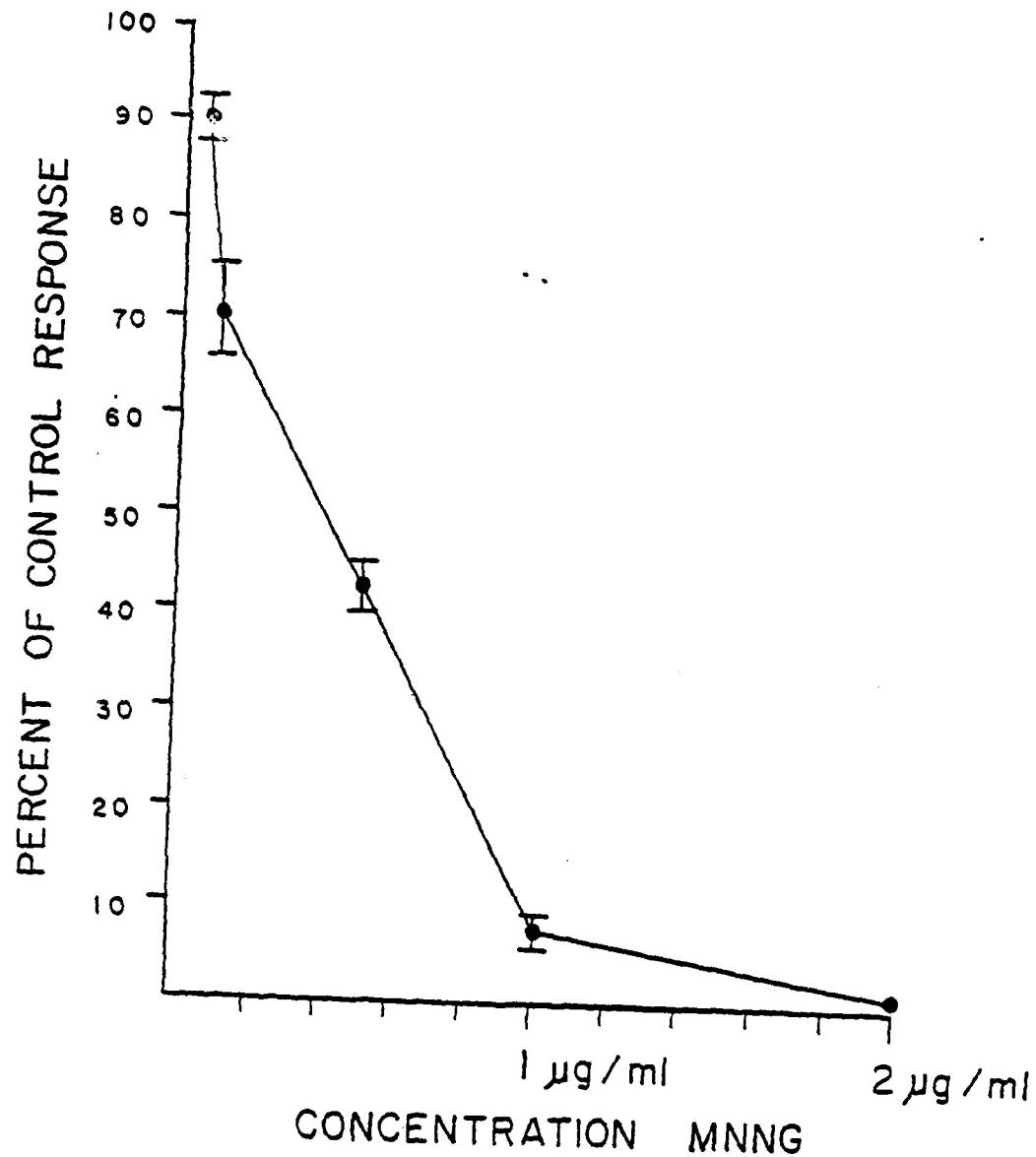
Figure 7. ST FeSV infected human skin fibroblasts. Twelve days post-infection (←) foci of infected cells. Phase contrast X 56.

Figure 8. Inhibition of ST FeSV transformation by benzo (a) pyrene.

D550 cells (1×10^5) were plated in 35 mm diameter wells with 4 ml of growth medium and incubated 18 h. (—) designated cells treated with BP before virus infection. (+) designated cells treated after virus adsorptionfor 24 h with BP as described in Materials and Methods. Cultures were washed and fed with growth media at the end of each treatment period and at 6 days post-virus infection. The cells were subsequently fixed and stained 3-4 days later. Virus induced foci were counted in non-treated and chemically treated wells. Horizontal line at 0 represents virus infected controls. Percentage inhibition was determined by:

$$100 = \frac{\overline{FFU} \text{ chemically treated}}{\overline{FFU} \text{ control}}$$

Significance was determined by Student's t-test. Concentrations of BP used were: $\triangle$. 15.0 µg/ml; $\circ$, 5.0 µg/ml; $\blacktriangle$, 1.0 µg/ml; +, significant inhibition.

BENZO(a)PYRENE AND STFeSV FOCUS FORMATION

△ – 15.0μg/ml
O – 5.0μg/ml
▲ – 1.0μg/ml
✳ – SIGNIFICANT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

Figure 9. Inhibition of ST FeSV transformation by N-acetoly-2-fluorenyl
Acetamide.

D550 cells (1×10^5) were plated in 35 mm diameter wells with 4 ml of growth
medium and incubated 18 h. Cells were treated with A-AAF as described in Fig.
8. Data were plotted as described in Fig. 8. Concentrations of A-AAF used
were: Δ, 1.0 µg/ml; o, 0.1 µg/ml; *, significant inhibition.

N-ACETOXY 2-FLUORENYL ACETAMIDE AND STFeSV FOCUS FORMATION

Δ - 1.0 μg/ml
O - 0.1 μg/ml
* - SIGNIFICANT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

Figure 10. Inhibition of ST FeSV transformation by aflatoxin B1.

D550 cells (1×10^5) were plated in 35 mm diameter wells with 4 ml of growth medium and incubated 18 h. Cells were treated with AFB1 as described in Fig. 8 . Percentage inhibition data was plotted as described in Fig. 8 . Enhancement was determined by dividing $\overline{FFU}$ treated cells by $\overline{FFU}$ control cells. Significance determined by Student's t-test. Concentrations of AFB1 used were: Δ , 1.0 μg/ml; o , 0.1 μg/ml; *, significant inhibition.

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AFLATOXIN B₁ AND STFeSV FOCUS FORMATION

$\triangle$ – 1.0 μg/ml
O – 0.1 μg/ml
* – SIGNIFICANT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)
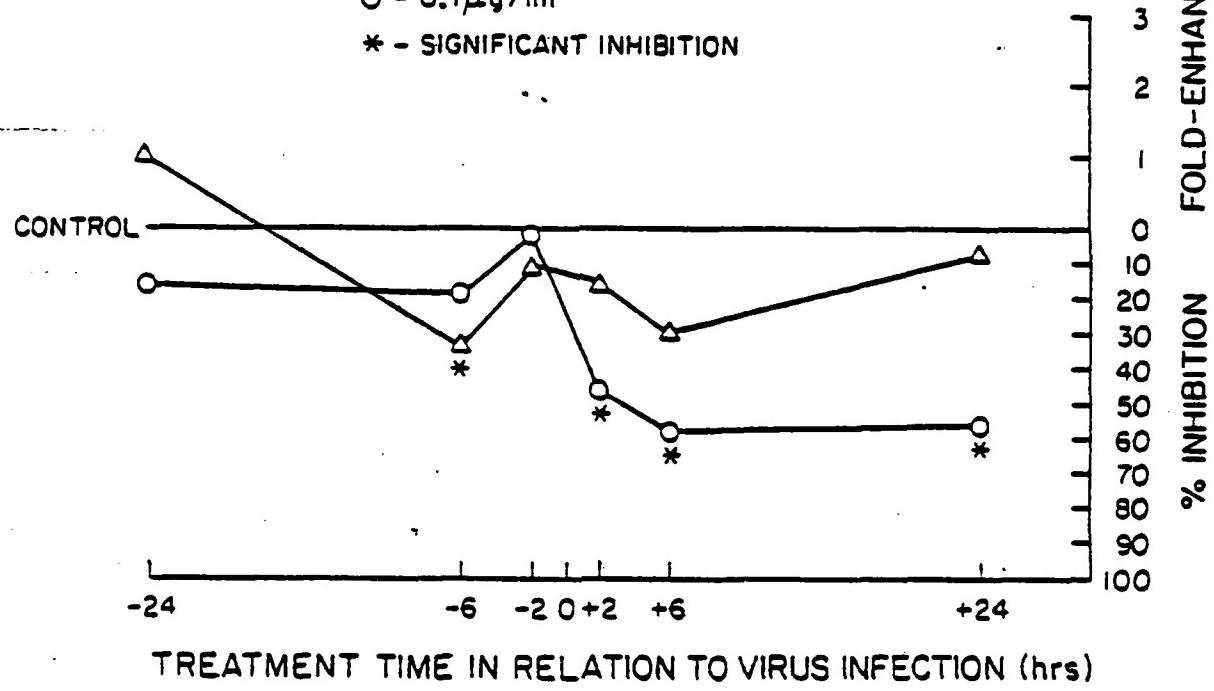
Legend Table 4:

Detroit 550 cells (1.5×10^6) were initially seeded into T-75 flasks and incubated 24 h. Medium was removed and cells infected as described in Materials and Methods. Ten days later, medium was replaced with 10 ml of growth medium and 24 h later, cells were harvested by either scraping or trypsinizing and processed as described below:

a) Cells removed with rubber policeman into the 10 ml of growth medium and q.s.'d to 20 ml with fresh growth medium and subjected to 1 cycle of freezing and thawing, and gross debris removed by 600 X g centrifugation. Two ml of clarified medium was used for infectivity assays and 18 ml used for reverse transcriptase assay.

b) Cells removed by trypsinizaiton, centrifuged, growth medium discarded and cells resuspended in 20 ml fresh growth medium and further processed as described in (a).

c) Cells removed by trypsinization and processed as described in (a).

d) Ten ml of supernatant frominfected flasks was q.s.'d to 20 ml with fresh medium and subjected to 1 cycle of freezing and thawing and further processed as described in (a).

e) Procedures previously described ( ). Rickard FeLV twice banded in sucrose run as standard in RT assay: 52,075 cpm.

Table 4: Methods of Cellular Harvest and DXM Treatment: Effect on Virus Synthesis.

Method of Harvest	FFU/ml (-)DXM	FFU/ml (+)DXM	Fold Increase	RT Assay[e] CPM (-)DXM	RT Assay[e] CPM (+)DXM	Fold Increase
Virus Infected Cultures						
Cells scraped + CF[a]	234	30,325	87X	8,848	77,427	9X
Cells trypsinized + CF[b]	0	192	(-)	8,464	102,874	12X
Cells trypsinized + CF[c]	233	23,300	100X	23,255	148,413	11X
CF Only[d]	344	19,125	56X	22,462	241,903	11X
Uninfected Cultures						
Cells scraped + CF	0	0	-	1,050	940	-
Cells trypsinized No CF	0	0	-	1,000	747	-
Cells trypsinized + CF	0	0	-	785	993	-
CF Only	0	0	-	595	806	-

ª - CF = Culture fluids.

Figure 11. Interaction and effect of Crocidolite asbestos on ST FeSV transformation.

Cells (1×10^5) were plated in 35 mm diameter wells with 4 ml growth medium and incubated 18 h. (–) designated cells treated with Crocidolite Asbestos before virus infection; (+) designates cells treated after virus adsorption for 24 h with asbestos suspended in HBSS. Cultures were washed and fed with growth medium at the end of each treatment period and at 6 days post-virus infection. Cells were subsequently fixed and stained 3-4 days later. Virus induced foci were counted in non-treated and asbestos treated wells. Horizontal line at 0 represents virus-infected control.

Percentage inhibition was determined by:

$$100 = \frac{\overline{\text{FFU}} \text{ asbestos treated}}{\overline{\text{FFU}} \text{ virus control}}$$

Fold enhancement determined by:

$$\frac{\overline{\text{FFU}} \text{ asbestos treated}}{\overline{\text{FFU}} \text{ virus control}}$$

Significance determined by Student's t-test (n = 24).

CONCENTRATION
▲ - 0.0001 μg/mℓ
● - 1.0 μg/mℓ
■ - 10.0 μg/mℓ
* - SIGNIFICANCE - 0.001-0.025
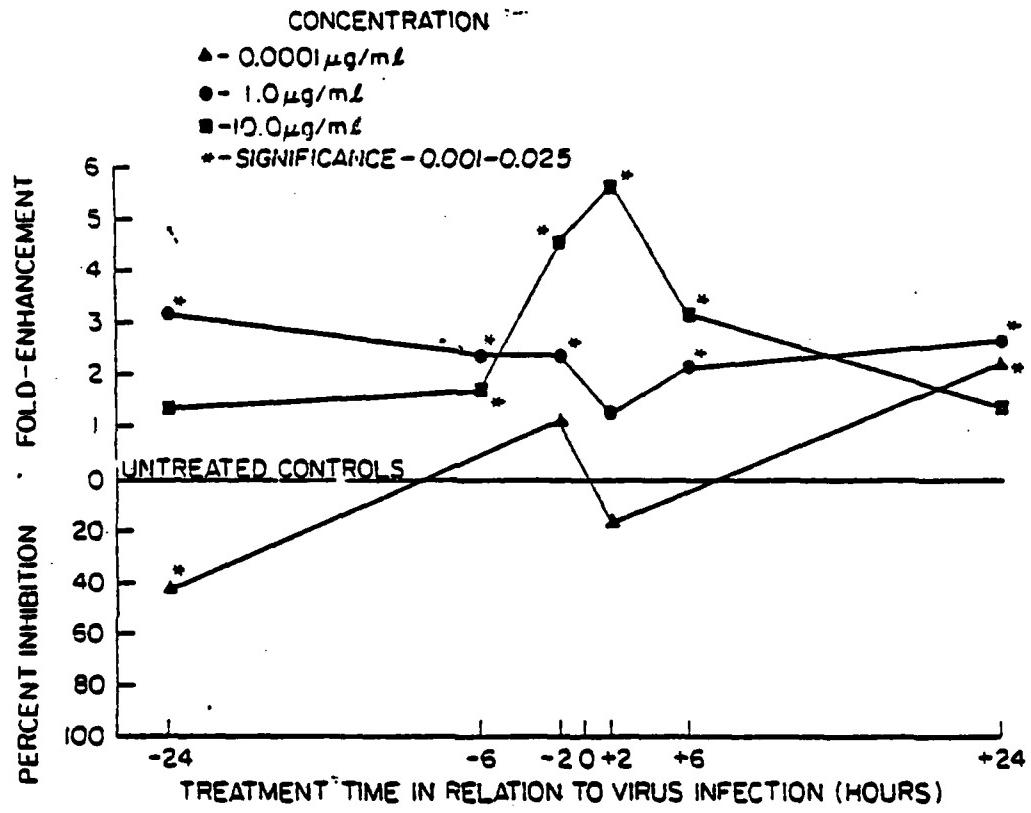
Figure 12. Time and dose related effect of Chrysotile asbestos on St FeSV transformation. Cells were treated with Chrysotile asbestos as described in Figure 11.

FOLD 10.5

CONCENTRATION
▲ -0.0001 µg/ml
● -0.001 µg/ml
■ -1.0 µg/ml
* - SIGNIFICANCE - 0.001 - 0.025

FOLD-ENHANCEMENT

10.5

4.0
3.0
2.0
1.0

UNTREATED CONTROL
0

PERCENT INHIBITION
20.0
40.0
60.0
80.0
100.0

-24   -6   -2 0 +2   +6   +24

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)

3u
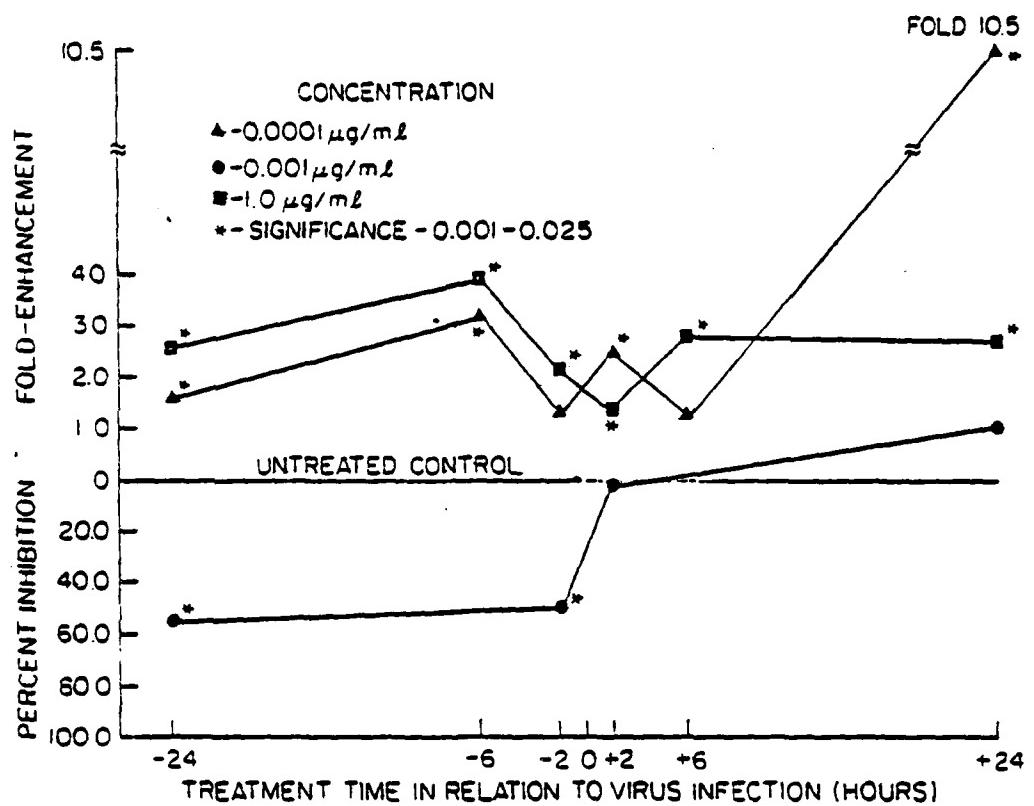
Figure 13. Inhibition of St FeSV transformation by Amosite asbestos. Cells were treated with Amosite asbestos as described in Figure 11.

CONCENTRATION
▲-0.0001 μg/ml
●-0.001 μg/ml
*-SIGNIFICANCE-0.001-0.025

UNTREATED CONTROL

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)
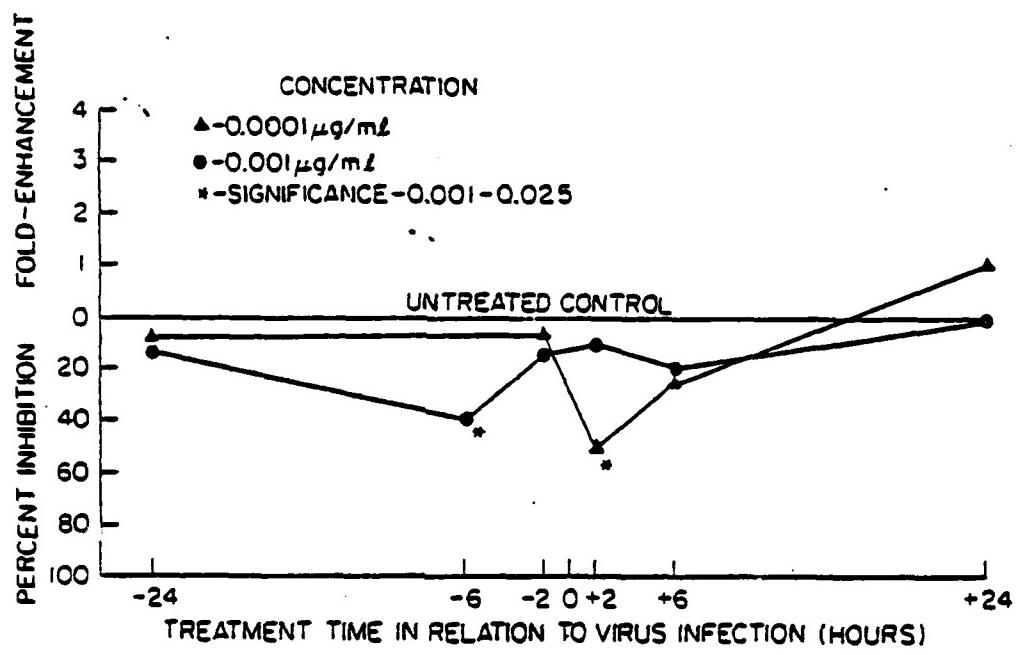
Table 6. Pre-Incubation of ST FeSV with Asbestos. Effect on Transformation.[1]

| Asbestos | Concentration µg/ml | Transformed Foci FFU/0.2 ml X 10-2 | | Effect on Transformation | (p) |
		Treated	Control		
Amosite	10	9.0± 2.6	22.5± 4.1	60%	0.001
Chrysotile	10	24.5± 4.2	22.5± 4.1	1.1	N.S.
Crocidolite	-10	23.5± 1.4	22.5± 4.1	1.1	N.S.

(a) = FFU ± S.D.

(b) = (p) significance determined by Student's t-test.

1 = 1.0 ml stock FeSV (1.0 X 10^5 FFU/ml) was incubated 30' at 30°C with 1.0 ml HBSS containing 10 µg of asbestos. Asbestos was removed by centrifugation, 2000 rpm for 15' at 4°C. Supernatants were aspirated and remaining virus titrated in D550 cells.

Table 7. Concomitant Treatment with Amosite, Chrysotile or Crocidolite Asbestos with Snyder-Theilen Feline Sarcoma Virus Infection.

| Asbestos | Concentration µg/ml | Transformed Foci FFU/0.2 ml X 10-2 | | Effect on Transformation | (p) |
		Treated	Control		
Amosite	10	51.9 ± 4.0[a]	22.5 ± 4.1	2.3↑	0.001[b]
Chrysotile	10	46.4 ± 4.8	22.5 ± 4.1	2.1↑	0.001
Crocidolite	10	42.5 ± 12.1	22.5 ± 4.1	1.9↑	0.050

(a) FFU = Focus Forming Units $\pm$ S.D.

(b) (p) = significance determined by Student's t-test.
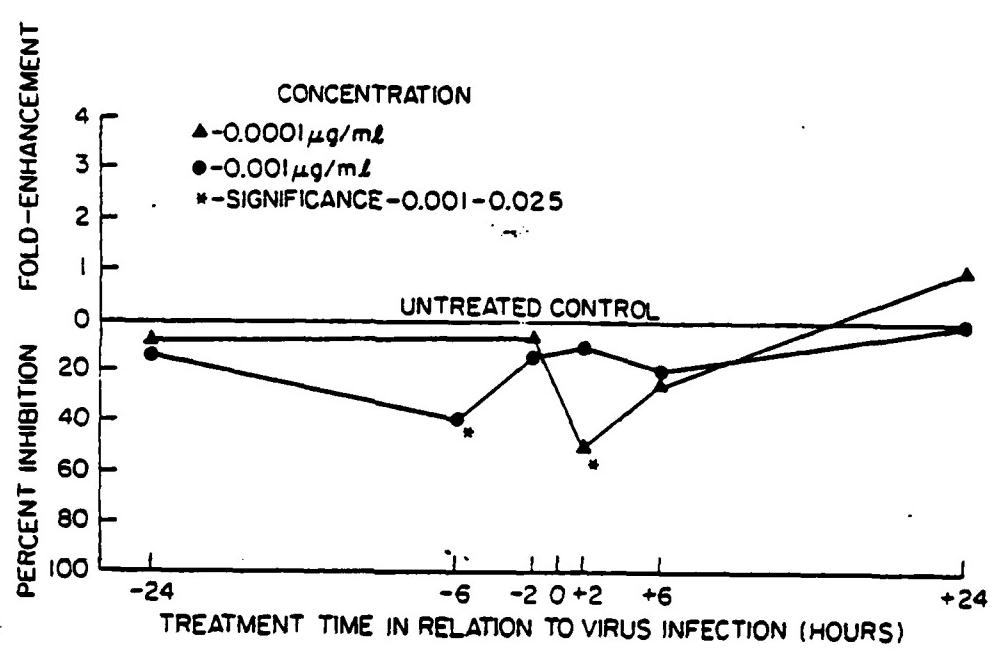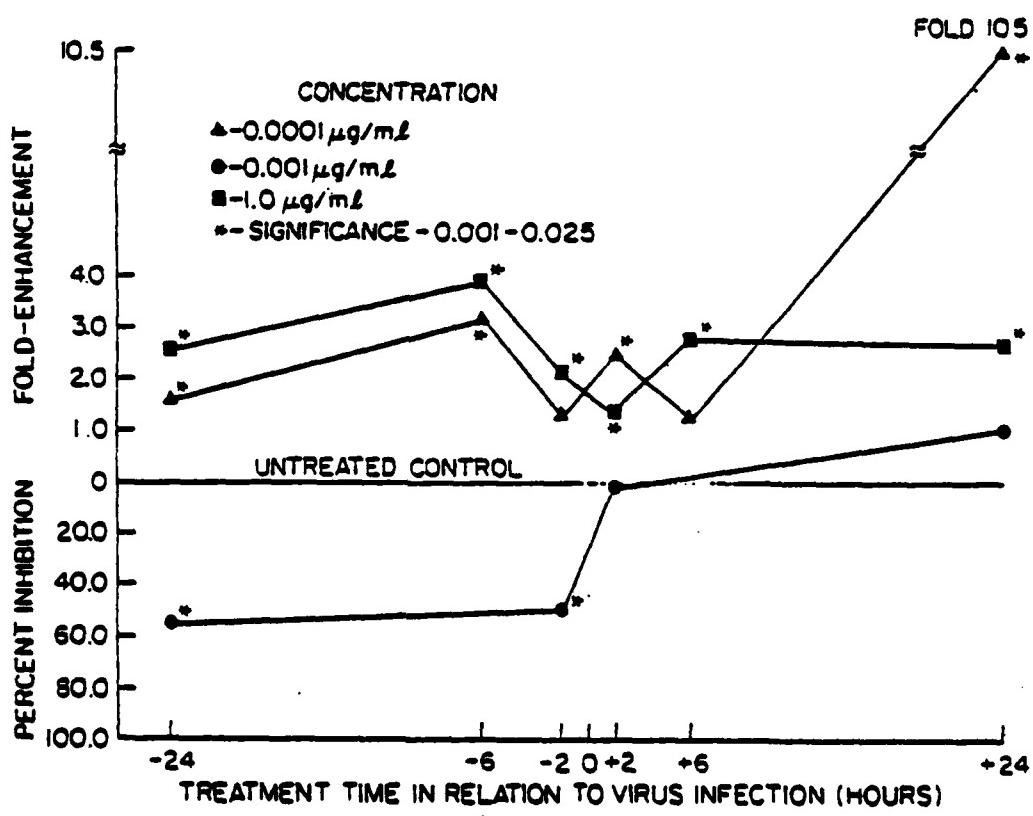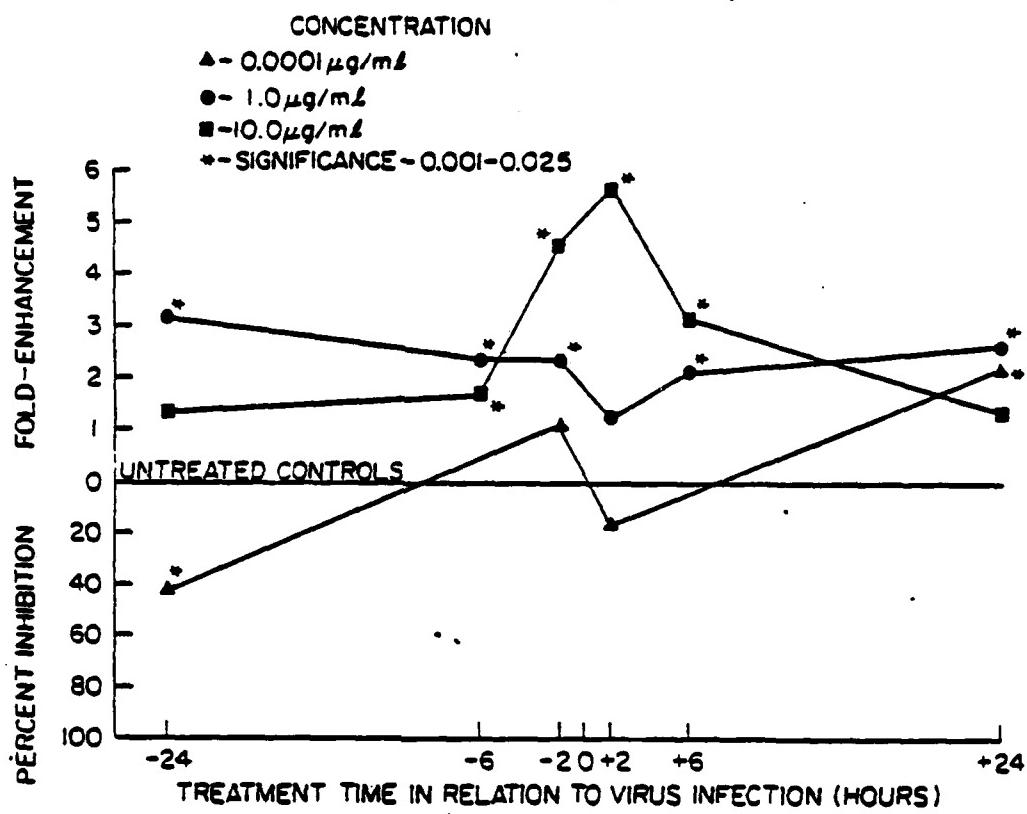
CONCENTRATION
▲-0.0001 μg/ml
●-0.001 μg/ml
*-SIGNIFICANCE-0.001-0.025

CONCENTRATION
▲ − 0.0001 μg/mℓ
● − 1.0 μg/mℓ
■ − 10.0 μg/mℓ
＊ − SIGNIFICANCE − 0.001 − 0.025

FOLD-ENHANCEMENT

UNTREATED CONTROLS

PERCENT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)

FOLD 105

CONCENTRATION
▲ − 0.0001 μg/mℓ
● − 0.001 μg/mℓ
■ − 1.0 μg/mℓ
＊ − SIGNIFICANCE − 0.001 − 0.025

FOLD-ENHANCEMENT

UNTREATED CONTROL

PERCENT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)

N-ACETOXY 2-FLUORENYL ACETAMIDE AND STFeSV FOCUS FORMATION

Δ – 1.0 μg/ml
O – 0.1 μg/ml
* – SIGNIFICANT INHIBITION
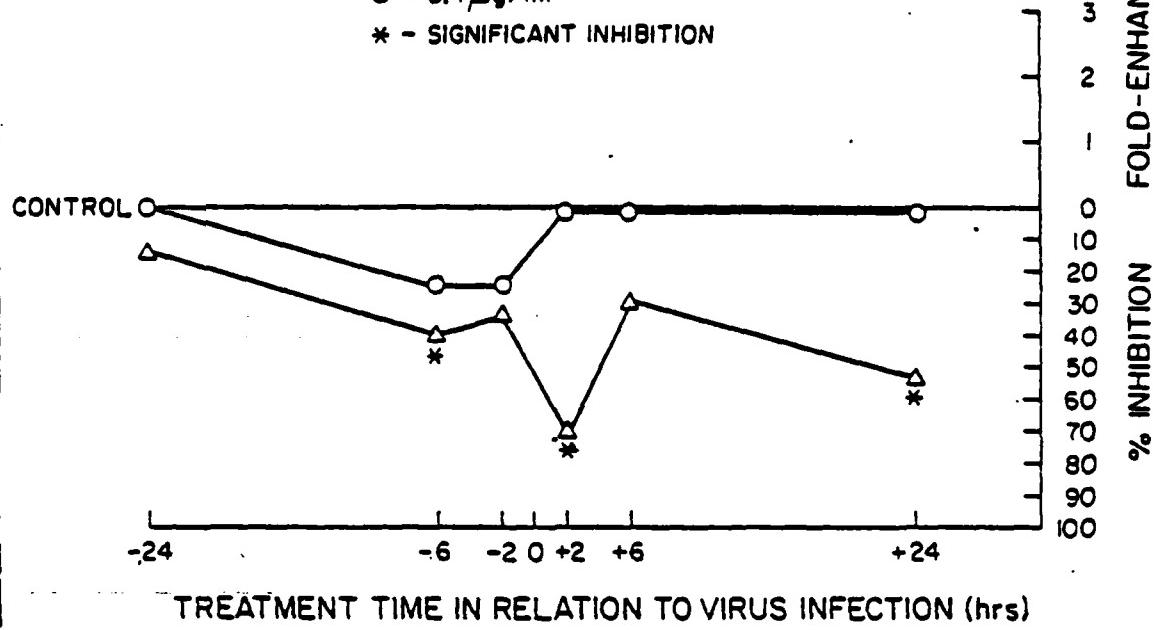
FOLD-ENHANCEMENT
% INHIBITION
CONTROL
TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

AFLATOXIN B₁ AND STFeSV FOCUS FORMATION

Δ – 1.0 μg/ml
O – 0.1 μg/ml
* – SIGNIFICANT INHIBITION
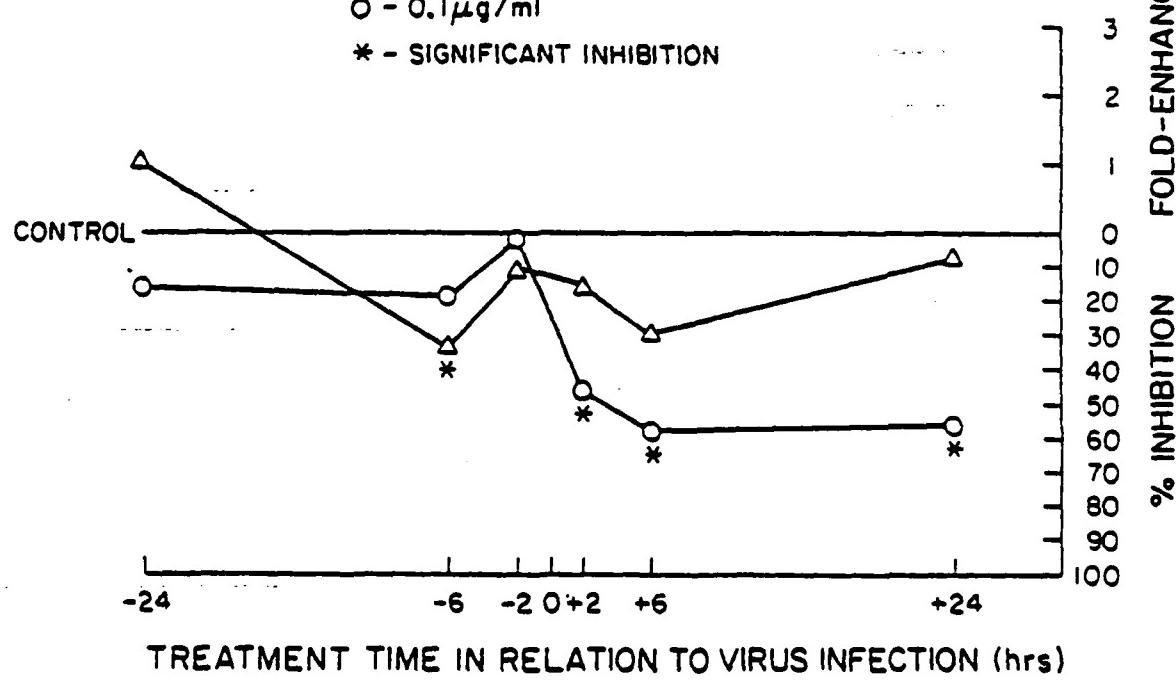
FOLD-ENHANCEMENT
% INHIBITION
CONTROL
TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

BENZO(a)PYRENE AND STFeSV FOCUS FORMATION

Δ - 15.0 μg/ml
O - 5.0 μg/ml
▲ - 1.0 μg/ml
∗ - SIGNIFICANT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)
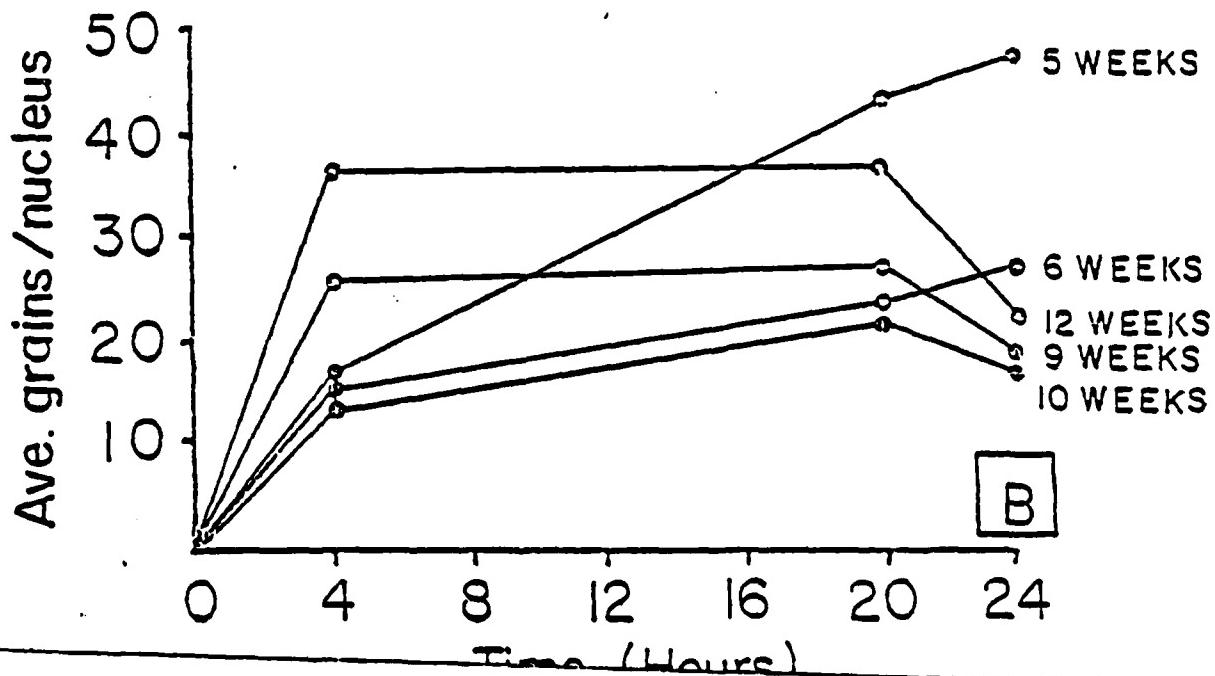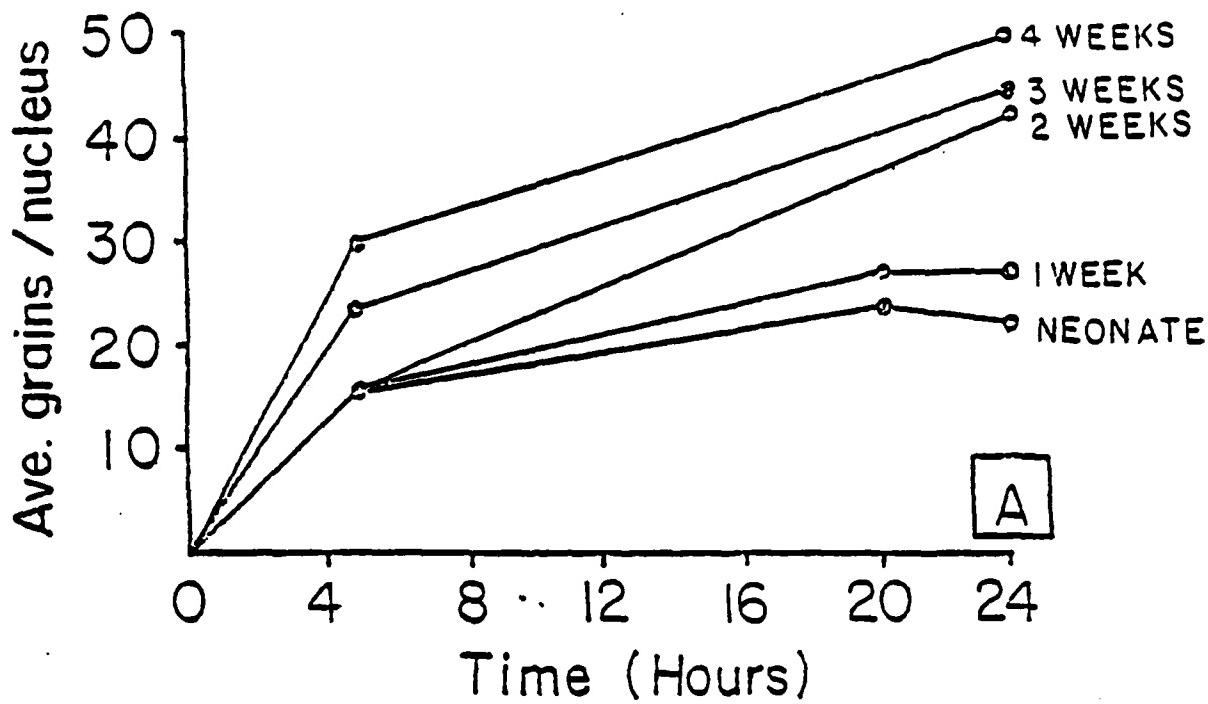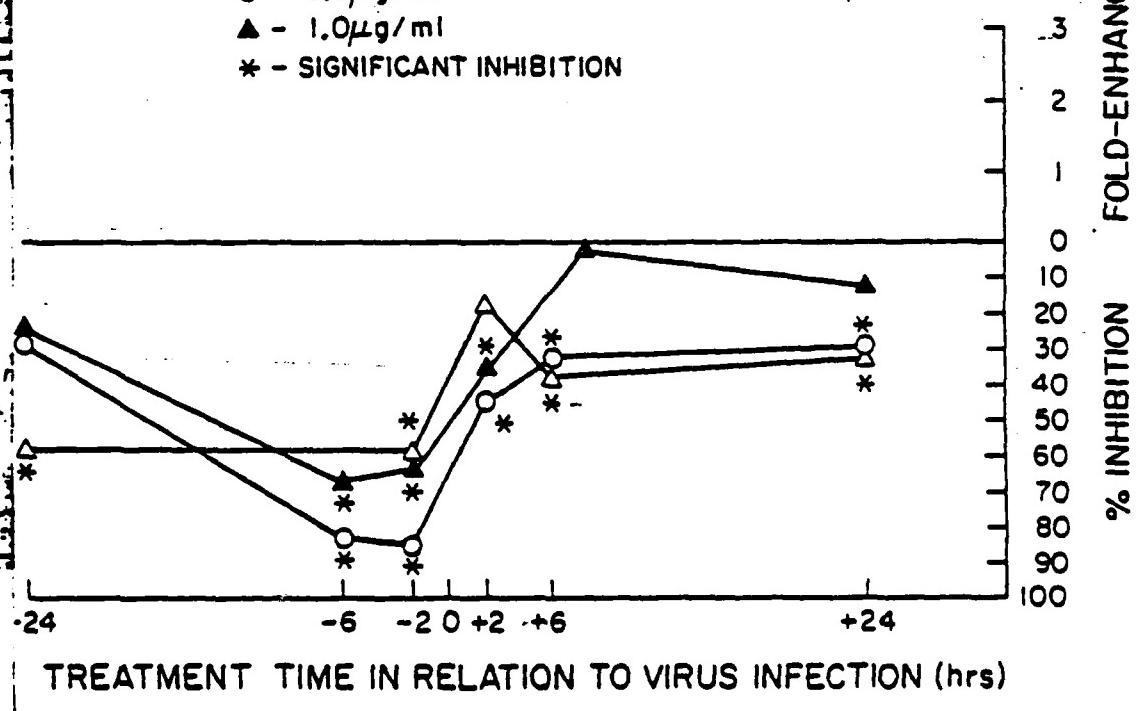
Figure 14. Excision Repair in Feline Fibroblasts from Different Aged Cats.

Feline fibroblast cells were grown from biopsied skin and used at passage 3. Cells were seeded on 1 X 3 sterile microscope slides and incubated 2 days at 37°C. Cells were washed in HBSS and incubated in Arginine deficient medium (ADM) plus dialyzed fetal bovine serum for 2 days. ADM containing 2 mm hydroxyurea was added to cells and incubated 1 h. Cells were washed and exposed to oVV light source at 100 ergs/mm^2. Cells were refed with ADM-HU medium containing 0.5 uCi ^{3}H-thymidine per ml and incubated at 37°C at 2, 6, 12, 18 and 24 hour intervals. Two control and 2 test slides were removed for fixation in methanol: acetic acid, rinsed 3X in 95% ethanol and air dried. In dark room, slides were dipped in 45C Kodak NTB2 nuclear track emulsion and dried for 20 minutes. Slides were placed in light-tight box with a dessicant and incubated 4-7 days at 4°C. Slides were developed with D-19 Kodak developer and stained with H and E. Fifty cells per slide were counted and averaged and background grain counts subtracted.

INDUCTION OF RETROVIRUS NON-PRODUCER HUMAN CELLS TO PRODUCER CELLS BY DEXAMETHASONE

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We previously reported human neonatal skin fibroblast cells treated with non-toxic concentrations of benzo (a) pyrene or aflatoxin B, inhibited feline sarcoma virus (FeSV) transformation.[1] Although comparable levels of reverse transcriptase (RT) activity were found in both chemically treated and untreated cultures, infectious virus was not recoverable from either.

Others have documented the stimulation and activation of murine and primate retroviruses and enhanced FeSV transformation of human cells by optimal concentrations of glucocorticoid hormones.[2-4]

The objectives of this study were to determine: 1) whether dexamethasone (DXM) induced both FeSV and FeLV synthesis in the apparent non-producer human cells and 2) whether feline oncornavirus-associated cell membrane antigen (FOCMA) and group-specific antigens (GSA) were synthesized in addition to RT, in non-DXM treated cells.

Snyder-Theilen strain FeSV[4] infected Detroit 550 neonatal human skin fibroblast cells produced very low levels of transforming virus when supernatant fluids from these cultures were titrated in normal D550 cells. The addition of 1.0 µg/ml DXM (9α-Fluoro-16α-methyl prednisolone) 24 hours post infection, significantly increased both FeSV and FeLV[5] synthesis when compared to non-DXM treated infected cells (Table 1). The increase in titer ranged from a 56-fold increase to a 100-fold increase for FeSV, while FeLV increased from 0 in untreated cultures to 6×10^4 FFU/ml in DXM treated cultures, a 2.6 fold excess when compared to FeSV.

RT activity (cpm of ^{3}H dTMP incorporated/reaction/hr)[1] likewise increased. The values ranged from a 9-fold increase in 1 experiment to a 11 fold increase in 2 other separate experiments.

GSA[6] and FOCMA[7] were detected in both FeSV infected DXM treated cells and FeSV infected cells only. Uninfected-cells, cell treated with 0.2% acetone, and/or DXM were negative.

TABLE I

DXM ENHANCED ST-FeSV AND FeLV SYNTHESIS IN D550 CELLS

Virus and Exp #	Virus Titer FFU/ml X 10^{-2}		Fold Increase
	(-) DXM	(+) DXM	
(1)	2.3	203.2	87X
(2)	2.3	233.0	100X
(3)	3.4	191.2	56X
Untreated Control	0	0	0
mean -	2.7 ± .5 mean -	209.2 ±17.6	P = <0.001*
FeLV^3	0	600.00	

* = Determined by Students "t" test.

Wu et al.[8] described retrovirus infected cells as falling into 3 phenotypic categories with respect to virus gene expression: (1) virus producer cells; (2) non-producer cells with partial gene expression (any virus, component); and (3) cells not having any detectable viral gene products. In this study, D550 cells infected with ST-FeSV could be classified as nonproducers with partial gene expression, i.e. transformation, reverse transcriptase, GSA and FOCMA antigens with little infectious virus being released. However, following DXM treatment, conplete viral gene expression resulted with infectious (transforming) virus released.

The fact that DXM permits complete oncogenic viral gene expression in human cells is significant since the phenotypic expression of most human transformed cells has formerly been shown to be category 2 (non-producer) with partial gene expression.[8,9]

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